Foraging behaviour of female Weddell seals (*Leptonychotes weddellii*) during lactation: new insights from dietary biomarkers

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List of Abbreviations

ACN	Acetonitrile
ANOSIM	Analysis of similarity
AsB	Arsenobetaine
BGT-1	Betaine/GABA transporter
BMHT	Betaine-homocysteine methyltransferase
CP	Crude protein
CSV	Comma separated file
CV	Coefficient of variation
DCM	Dichloromethane
DMG	*N*,*N*-dimethylglycine
DMS	Dimethylsulfide
DMSP	Dimethylsulfoniopropionate
DPP	Days post partum
ED	Energy density
EL	Early lactation
ESI	Electrospray ionisation source
FMO	Flavin monooxygenase
GB	Glycine betaine
GC-MS	Gas chromatography-mass spectrometry
GF-AAS	Graphite furnace-atomic absorption spectrometry
HPLC	High performance liquid chromatography
HS-GC	Headspace-gas chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD_b	method limit of detection
LOD_s	sample limit of detection
LOQ_s	sample limit of quantitation
LL	Late lactation
MeOH	Methanol
ML	Mid lactation
MRM	Multiple reaction monitoring
MDS	Non-metric multidimensional scaling

^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
PC	Proximate composition
PCA	Perchloric acid
QC	Quality control
QFASA	Quantitative fatty acid analysis
PAR	Peak area ratio
RPC	Reverse phase chromatography
SIMPER	Similarity of percentages
TDR	Time depth recorder
TMA	Trimethylamine
TMAO	Trimethylamine *N*-oxide

Abstract

Despite extensive studies on Weddell seals (*Leptonychotes weddellii*) in McMurdo Sound since the 1960s, uncertainty still remains regarding female foraging habits during the lactation period. Based on their large body mass at the start of lactation and large relative mass loss at the end, the current hypothesis is that Weddell seals fast or feed to a neglible extent during lactation. However, this hypothesis has not been fully tested to date, as evidence for foraging is indirect and is based primarily on dive behaviour. The work presented in this thesis describes the development of a new dietary method, the *biomarker method*, and its application for studying the foraging behaviour of female Weddell seals during lactation. Biomarkers were used to (1) monitor the onset of feeding in individual animals, and (2) determine what prey females were feeding on using characteristic/taxon-specific biomarker patterns. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays were developed to detect and quantify dietary biomarkers in biological samples, mainly tissues, serum and plasma. Trimethylamine *N*-oxide, arsenobetaine, dimethylsulfoniopropionate, homarine and glycine betaine were first measured in thirty-three prey and potential prey species of Weddell seals collected from the Ross Sea and McMurdo Sound regions of Antarctica. These same compounds were then measured in the plasma of twelve female Weddell seals over the lactation period at the Hutton Cliffs seal colony, McMurdo Sound in 2006. Time-depth recorders monitored seal dive activity over the same period.

The data obtained from both NMR and LC-MS/MS assays showed that biomarkers in Antarctic species varied both in content and concentration. The compound homarine, which occurs primarily in cephalopods, is suitable for distinguishing between major food groups of known prey of Weddell seals (*i.e.*, fishes *versus* cephalopods). DMSP, a compound that occurs primarily in fish common in McMurdo Sound (*e.g.*, *Trematomus bernacchii* and *Pagothenia borchgrevinki*) but not in significant amounts in *Dissostichus mawsoni* or *Pleuragramma antarcticum*, two main prey items for Weddell seals, may also be a suitable biomarker for distinguishing between major and minor prey types. The detection of plasma TMAO, AsB and homarine indicated that 75% of Weddell seals studied fed during lactation. The presence of these three compounds indicates the seals were preying upon a combination

of fish and cephalopods. Two lactating females started foraging as early as 9 to 12 days postpartum and elevated biomarker levels were concurrent with increased dive activity. The onset of foraging and dive behaviour amongst individuals was highly variable; however, the results suggests that the number of females who feed during lactation may be more prevalent and initiated at an earlier stage than previously thought. This may have implications for future reproductive success given effects of climate change on sea ice abundance and resource availability.

Overall, the work presented in this thesis provides new insights into the foraging behaviour of female Weddell seals during lactation and has added to the current knowledge of the biomarker distribution within the Antarctic ecosystem.

Chapter One

Introduction and Literature Review

Female Weddell seal and her pup shortly after birth. Photo by C. Lenky.

1.1 Brief overview

Marine mammals forage at widely different spatial and temporal scales, often at deep depths and in remote locations. The inability to confirm underwater prey encounters has led researchers to develop a number of different techniques for determining diet in marine mammals. Each method has its own advantages and disadvantages, which are discussed in the following section.

A new method for exploring diet in marine mammals is the *biomarker method*. The principle behind the biomarker method is the detection of metabolites in mammalian body fluids such as urine or plasma. Eisert et al. (2005) pioneered the use of *marine osmolytes* and their analogues as biomarkers. Within this family of compounds, any osmolyte not synthesised by a mammalian predator represents a potential dietary biomarker, since it should only occur in the predator as a result of food intake (Eisert et al. 2005). Since osmolytes and their analogues are often found in high concentrations (millimolar or higher) in prey species, they should be measurable in the blood of the consumer after feeding.

Suitable compounds identified by Eisert et al. (2005) were the osmolyte trimethylamine *N*-oxide (TMAO) and the osmolyte analogue, arsenobetaine (AsB). They applied the biomarker method to the Antarctic Weddell seal (*Leptonychotes weddellii*) in order to determine whether females were feeding or fasting during the lactation period. Presently, the biomarker method gives an indication as to whether a female Weddell seal is feeding or fasting, but a key question is whether osmolytes vary sufficiently among different prey taxa to be useful biomarkers to detect consumption of particular prey types. A prey-specific biomarker method would be useful to researchers because it would allow them to obtain diet information repeatedly without significantly harming the animal. At present, stomach analysis is the only method that can definitively describe diet, but being terminal, this method cannot be applied to many seals at once nor can it be applied to protected seals such as the Weddell seal.

The overall goal was to further develop the dietary biomarker method through the development of analytical assays that could be applied to studying the foraging behaviour of not only pinnipeds, but other marine mammals or sea birds. The lactating Weddell seal was used as a model species in order to build upon and expand on the previous work of Eisert et al. (2005). The aim of this chapter is to 1) review the current methods used to study the diet of marine mammals; 2) introduce the biomarker method; 3) review what is currently known

about osmolytes, and 4) review the general biology and ecology of the Weddell seal and the application of the biomarker method to lactating females. Thesis aims are presented at the end of this chapter.

1.2 Methods for studying the diet of marine mammals

Methods that researchers employ to study the diet of marine mammals are varied, and include "traditional" methods such as the analysis of stomach contents and faeces (Lawson et al. 1995, Lake et al. 2003) or "new" methods such as stable isotope analysis of various tissues (Newsome et al. 2010), DNA extraction from faeces (Deagle et al. 2005) and fatty acid signature analysis of lipids (Iverson et al. 2004). Other methods that are used include the use of dive profiles (Castellini et al. 1992, Harcourt et al. 2000), stomach temperature probes and jaw sensors (Andrews 1998, Plötz et al. 2001); however, these methods provide limited information on actual diet and only a limited number of individuals can be studied. These methods are not reviewed here, but are discussed elsewhere in the thesis.

1.2.1 Stomach and faecal analysis

Identifying prey hard parts from stomach contents and faeces is one of the most commonly used techniques for studying the diet of marine mammals (Olesiuk 1993, Hammond et al. 1994, Lawson et al. 1995, Lawson and Stenson 1997, Lake et al. 2003, Orr et al. 2004, De Pierrepont et al. 2005, Spitz et al. 2011). Hard part analysis is inexpensive, and the numerical abundance, length and weight of prey can be calculated using species-specific regression equations (Lawson et al. 1995). However, a detailed reference collection of fish otoliths (ear bones), cephalopod beaks or other diagnostic hard parts are required for accurate species identification.

There are well known biases associated with hard part analysis (Murie and Lavigne 1985, Harvey 1989, Pierce and Boyle 1991, Tollit et al. 1997). One difficulty is that rates of erosion of fish hard parts differ across both fish and pinniped species (Tollit et al. 1997, Bowen 2000). Due to the short retention time of food remaining in the digestive system, diet information is limited to a short temporal scale. Prey items with more robust hard parts that survive digestion have a high probability of detection and identification in stomach and faecal analysis, whereas prey with less robust or no hard parts erode at a faster rate and are likely to be under represented or missed entirely (Pierce and Boyle 1991, Tollitt et al. 1997). For

example, in the case of the Antarctic toothfish *Dissostichus mawsoni*, Weddell seals only consume muscle and viscera (Ainley and Siniff 2009). In contrast, cephalopod beaks are not digested, but tend to accumulate in stomachs resulting in underestimation of the contribution of cephalopods in faecal analysis but not stomach analysis (Bigg and Fawcett 1985, Gales and Cheal 1992, Staniland 2002). Additionally, cephalopod beaks can be regurgitated by seals and thus not show up in stomach or faecal analysis (Childerhouse et al. 2001, Mcintosh et al. 2006). In the case of partially digested otoliths, it is often possible to identify the prey even though morphometric data cannot be used to estimate the length or weight of the prey. A number of studies on captive pinnipeds have aimed to reduce these biases by examining otolith digestion rates and calculating numerical correction factors which adjust the results for otolith erosion in faecal analysis (Tollit et al. 1997, Tollit et al. 2004, Grellier and Hammond 2006, Tollit et al. 2007, Phillips and Harvey 2009). Recovery rates were found to vary amongst pinniped species and also between individuals. Recovery also varied with the size and shape of otoliths (Tollit et al. 2007) and seal activity (Tollit et al. 2003). Thus, it would be difficult to apply correction factors calculated from captive animals to wild animals, which are more active and are likely to consume more diverse prey.

The majority of studies examining stomach contents come from cetaceans or pinnipeds that have been stranded on beaches, by-caught in fisheries or purposely killed (Lawson et al. 1995, De Pierrepont et al. 2005, Meynier et al. 2009, Spitz et al. 2011). Stomach contents of animals found dead on beaches may not represent the entire population as they may have had health issues, while animals caught in commercial fishing gear may have a greater proportion of the targeted species in their stomachs. Direct killing of animals for stomach contents only produces representative results if using a large number of individuals (Nilssen et al. 1995, Haug et al. 2004). Additionally, direct killing is illegal in most parts of the world, except where animals are hunted for subsistence (Hovelsrud et al. 2008). Faeces are easy to collect from animals that haul out on land, but are harder to obtain from species that do not, such as the harp seal *Phoca groenlandica* which only hauls out on pack-ice to give birth, and cetaceans. In most cases it is not possible to assign faeces to individuals.

Regardless of the biases associated with hard part analysis, it is the only method by which the approximate number of prey species and size of the prey can be determined.

1.2.2 Molecular analysis

Over the last decade, molecular tools have been developed to identify DNA of particular prey species in the soft material of pinniped faeces (Deagle et al. 2005, Parsons et al. 2005, Casper et al. 2007, Deagle and Tollit 2007, Matejusova et al. 2008, Pompanon et al. 2012). While this method typically requires the design of specific markers (although newer technologies do not necessarily (Pompanon et al. 2012), and relies on prior knowledge of DNA sequences of all potential prey species of the consumer, it can identify species that are often underestimated in faecal hard-part analysis due to digestion or retention in the gut (*e.g.*, invertebrates, fragile otoliths and cephalopod beaks). Deagle et al. (2005) and Casper et al. (2007) found that in feeding experiments with captive animals, the probability of detecting a prey type was higher with DNA analysis compared with hard part analysis.

1.2.3 Stable isotope analysis

Stable isotope ratios of carbon and nitrogen ($\delta^{13}C$, $\delta^{15}N$) are widely used in marine mammal studies to study trophic relationships and ecosystem dynamics (Lawson and Hobson 2000, Cherel et al. 2008, Cherel et al. 2009, Newsome et al. 2010, Arnould et al. 2011, Witteveen et al. 2011). Because nitrogenous waste products containing the "lighter" ^{14}N isotope are preferentially excreted (Hobson et al. 2004a), consumer tissues are enriched in ^{15}N relative to their food and $\delta^{15}N$ measurements indicate a consumer's trophic position. In contrast, $\delta^{13}C$ values vary little along the food chain and are mainly used to determine sources of primary production. In the marine environment, $\delta^{13}C$ values indicate whether a consumer is foraging inshore *versus* offshore, or in pelagic *versus* benthic habitats (Kelly 2000, Kurle and Worthy 2002). Stable isotope ratios can be determined in various tissues, and the time-scale of foraging covered by the analysis depends on the biochemical turnover rate of the isotope in the tissue being studied. For example, blood plasma and serum, skin and muscle have turnover times of weeks to months (Burns et al. 1998, Kurle 2002, Kurle and Worthy 2002, Alves-Stanley et al. 2010); hair and vibrissae turn over within a year to several years (Hirons et al. 2001, Cherel et al. 2009); and bones, teeth or baleen have turnover times of years to decades (Best and Schell 1996, Hobson et al. 2004a, Hobson et al. 2004b, Newsome et al. 2007). While the stable isotope method is useful for determining foraging location and trophic position, the number of individual prey being consumed cannot be estimated.

1.2.4 Fatty acid signature analysis

A large number of recent studies have employed the use of fatty acids or quantitative fatty acid signature analysis (QFASA) in dietary studies on marine mammals (Lea et al. 2002b, Beck et al. 2007a, Beck et al. 2007b, Ridoux et al. 2007, Wheatley et al. 2007, Thiemann et al. 2008, Tucker et al. 2009a, Tucker et al. 2009b). The principle behind this method is that long-chain fatty acids in prey species deposit into the adipose (fat or blubber) tissue of the predator and that this provides a record of dietary intake over periods of several weeks to months (Budge et al. 2006). The common forms of fatty acids in marine biota are phospholipids, wax esters and triacylglycerols (Budge et al. 2006). Blubber is most often the tissue of choice for fatty acid studies on free-ranging marine mammals because it can be obtained from biopsy after an animal is captured or through dart sampling of large whales and cetaceans. Some studies have also used milk as a source of fatty acid analysis, the idea being that milk fatty acids derive from nutrients acquired while foraging (Lea et al. 2002b, Staniland and Pond 2005, Baylis et al. 2009, Baylis and Nichols 2009). QFASA, the quantitative application of fatty acid analysis, was first utilised by Iverson et al. (2004). QFASA uses a mathematical model to estimate the proportion of different prey which first requires a fatty acid library of all potential prey of the predator. Calibration coefficients are also needed for individual fatty acids which take into account lipid metabolism and relative deposition of the fatty acids in the predator's adipose tissue. Calibration coefficients are calculated from captive animals that are fed known diets (Iverson et al. 2004, Iverson et al. 2007, Nordstrom et al. 2008). Some of the drawbacks to using fatty acid analysis are that blubber is not a uniform tissue and stratification exists in some cetaceans (Smith and Worthy 2006, Koopman 2007) and pinnipeds (Arnould et al. 2005, Wheatley et al. 2007), and that lipids are not deposited or metabolised uniformly along the body (Arnould et al. 2005). Therefore, it is necessary to sample the entire blubber layer (inner and outer layers) as well as selecting an area where fatty acids are deposited. There are additional challenges associated with determining how dietary fatty acids are metabolised and whether certain fatty acids are selectively deposited or mobilised in the predator's tissue. Calibration coefficients also differ amongst predators (Iverson et al. 2004, Tollit et al. 2006), which limits the use of QFASA to those species for which calibration coefficients exist.

All of the methods mentioned above have their pros and cons, and a number of researchers have taken to combining dietary techniques to take advantage of complementary information

delivered by different methods (Lea et al. 2002b, Hammill et al. 2005, Staniland and Pond 2005, Baylis and Nichols 2009). Choosing an appropriate dietary method also depends on the spatial and temporal scale of foraging that is being studied, as well as a number of intrinsic factors such as age (pups, juveniles or adults), sex (males *versus* females), season, life-cycle stage (breeding season or migration) and protection status (permission of invasive techniques/access to animals). One area that has received considerable attention is the lactation stage of pinnipeds.

1.3 Pinniped lactation strategies

Maternal investment during lactation is the most energetically expensive period in a mammal's life cycle (Oftedal et al. 1987b). In pinnipeds, lactation strategies range from complete dependence on body reserves to support lactation (a "capital strategy", such as in most large-bodied phocids[1]) to maternal foraging and reliance on food consumption (an "income strategy", such as in otariids[2] and some smaller phocid species). The lactation period in otariids ranges from four months in the northern fur seal *Callorhinus ursinus* to three years in the Galapagos fur seal *Arctocephalus galapagoensis* (Bonner 1984). Otariids alternate between nursing their pups on shore and foraging at sea. The length of lactation is influenced by the availability of food, while foraging trip duration is influenced by the distance between breeding site and food source (Oftedal et al. 1987a). Amongst phocids, lactation ranges from just four days in hooded seals *Cystophora cristata* (Bowen et al. 1985) and up to 119 days in the Mediterranean monk seal *Monachus monachus* (Aguilar et al. 2007), with a median duration of *c*. 4 weeks (Eisert 2003, Oftedal 2011). The relatively short lactation period of phocids means that a large amount of energy must be transferred to the pup in a short time and that phocids produce milk that is extremely rich in fat (Oftedal et al. 1988).

Lactation strategies do not strictly follow the taxanomic division between phocid and otariid seals. In many otariids, females fast for approximately one week after giving birth, whereas a number of phocid seals feed during lactation. Probably the best known example of a phocid with an income strategy is the harbour seal *Phoca vitulina* which starts feeding during mid–lactation (Boness et al. 1994). Bowen et al. (2001) found that maternal mass was an important

[1] Family Phocidae: true seals (no external ears)

[2] Family Otariidae: fur seals and sea lions (eared seals)

factor influencing lactation performance and that smaller females were not able to store sufficient energy stores in their blubber and required supplementary feeding to support milk production. Other phocids, including the harp, ringed *Pusa hispida*, bearded *Erignathus barbatus* and ice breeding grey seal *Halichoerus grypus*, may employ mixed capital and income strategies (Hammill et al. 1991, Lydersen and Kovacs 1996, 1999). Since food is the principal energy source for lactation, foraging may be necessary for smaller females to replenish body stores used in milk production during the latter stages of lactation.

Weddell seals have one of the longest lactation periods amongst phocid seals, lasting for approximately 33 to 53 days (Kaufman et al. 1975, Thomas and Demaster 1983b). Females face considerable energetic demands from their pups which grow rapidly from 30 kg at birth to *c.* 80-120 kg at weaning (Bryden et al. 1984, Tedman and Green 1987). Weddell seals have often been considered to follow a capital breeding strategy due to their large postpartum body mass of 400 to 450 kg (Tedman and Green 1987), which is four to five times greater than the harbour seal (85 kg, Bowen et al. 2001) and the extreme weight loss observed in lactating females of approximately 40% postpartum body mass (Eisert and Oftedal 2009). It is not clear whether females have accumulated sufficient energy stores prior to parturition to support maternal maintenance plus milk production throughout the entire breeding season. There is evidence in the literature to suggest that females may indeed feed in order to replenish depleted energy stores (Testa et al. 1989, Hindell et al. 2002, Eisert et al. 2005, Wheatley et al. 2008).

1.4 Foraging in lactating Weddell seals

The Weddell seal is one of the largest species of true seals and also the southernmost species of mammal. Their distribution is circumpolar and they are associated with the land-fast ice around the Antarctic continent and sub-Antarctic islands. In the Ross Sea, approximately 10,000 Weddell seals are distributed along the Victoria Land coast, from Cape Roget in the north to McMurdo Sound in the south (Siniff and Ainley 2008). One of the largest concentrations is located in Erebus Bay with a current population of approximately 4,000 individuals (Siniff and Ainley 2008) (Figure 1.1). In Erebus Bay, individual seals are identified by hind flipper tags as part of a long–term tagging study and most are of known age (Cameron and Siniff 2004). Weddell seals display a high site fidelity and philopatry, returning to traditional sites each austral spring to breed. Males and non-lactating females

aggregate in these colonies to mate during the latter portion of the lactation period. After weaning, seals disperse north into the Ross Sea or remain within Erebus Bay (Burns et al. 1999, Stewart et al. 2000).

The breeding season for Weddell seals starts in the austral spring of each year when pregnant females arrive at pupping colonies. Pupping occurs soon after the females haul out on the fast ice and pups are born between September and November depending on latitude (Stirling 1969). The majority of pups are born between mid-October and early November in McMurdo Sound (Kaufman et al. 1975). The location of pupping colonies (Figure 1.1) is determined by tidal cracks that form along the base of cliffs or islands that allow Weddell seals to maintain breathing holes in the fast ice (Stirling 1969). In McMurdo Sound, the largest pupping colonies occur on multi-year ice that denies access to potential predators such as killer whales *Orcinus orca* and leopard seals *Hydrurga leptonyx*.

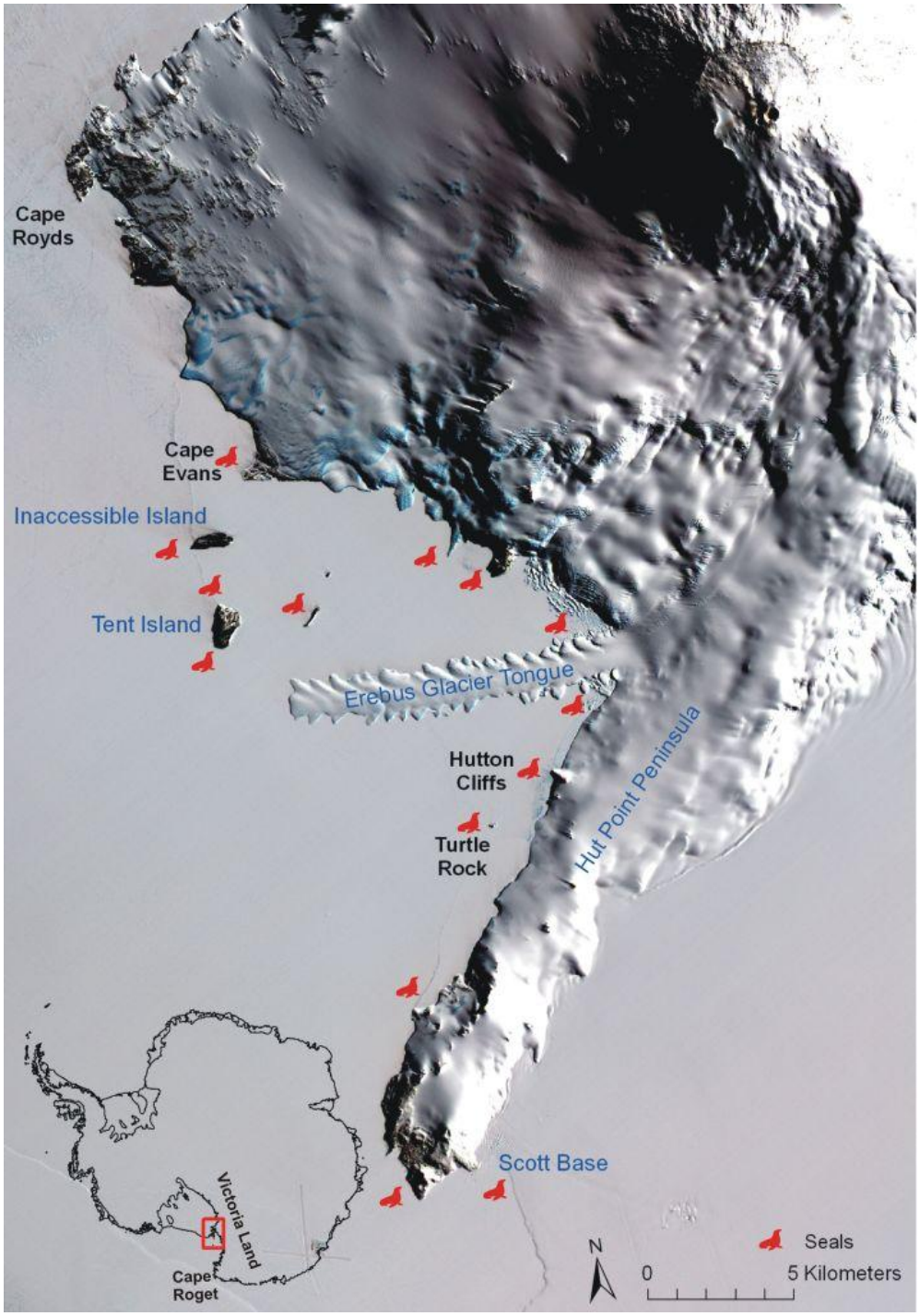


Figure 1.1. Weddell seal breeding colonies in Erebus Bay, Antarctica and the location of Hutton Cliffs (77°51'S, 166°45'E). Figure based on Cameron et al. (2004).

Not surprisingly, detecting food consumption in lactating Weddell seals is difficult. It would be easy to determine if females were fasting if they remained solely on the ice, but females commence diving two weeks after parturition and dives typically increase in frequency and

length later on in lactation (Thomas and Demaster 1983a). Evidence for foraging in female Weddell seals is indirect and is based primarily on dive behaviour (Testa et al. 1989, Hindell et al. 2002, Sato et al. 2002, Sato et al. 2003) and maternal mass changes (Tedman and Green 1987, Testa et al. 1989), with one study based on fatty acids in blubber (Wheatley et al. 2008). Blubber fatty acids are not suitable for examining female's diets during lactation because blubber sampled at the start of lactation will reflect food consumption in the winter months prior to giving birth. Blubber fatty acids do not allow for fine-scale examination of feeding over the lactation period. Time-depth recorders (TDRs) provide information on the depth, duration and location of dives. The shape of time depth profiles have been used to classify dives into categories such as U-shaped, V-shaped, round and flat bottom and then functions such as foraging, exploration or transiting have been attributed to these dive categories (Schreer and Testa 1995, 1996). These studies have provided a better understanding about Weddell seal movements underwater, but this method does not have the ability to confirm underwater prey encounters or feeding bouts. There is also the possibility that foraging may occur during types of dives not classified as feeding dives. Studies that have employed video or still cameras to observe hunting behaviour have primarily been of males or non-lactating females at holes far away from breeding colonies (Davis et al. 1999, Davis et al. 2003, Watanabe et al. 2003). Only two studies have monitored lactating females using digital cameras and with conflicting results. Sato et al. (2002) concluded that lactating females forage during dives greater than 50 m based on neck stretching behaviour. In a separate study these authors examined diving behaviour in mother/pup pairs, but found no evidence of feeding (Sato et al. 2003). However, only five females were examined in the latter study compared to forty-one females in Sato et al. (2002).

In order to determine what proportion of lactating females depend on food intake to be able to complete lactation successfully, it is first necessary to be able to detect foraging. To date, no single method has shown definitive food consumption in lactating Weddell seals except for the biomarker method presented by Eisert et al. (2005). Similar in principle to fatty acid and stable isotope analysis, this approach allows food intake to be confirmed at a specific point in time from the presence of dietary biomarkers in body fluids.

1.5 The biomarker method

Compounds that are absorbed intact from prey but are not synthesised by the predator are suitable as dietary biomarkers. Compounds found in possible prey species that meet this criterion include marine osmolytes and their analogues.

1.5.1 Marine osmolytes

Marine organisms have developed a number of adaptive strategies to cope with environmental stresses. Most marine organisms (*e.g.*, invertebrates) are osmoconformers in that their body tissues are approximately iso-osmotic with seawater (1000 mOsm). Marine animals that are not osmoconformers (*e.g.*, teleosts) are hypo-osmotic to their environment with blood concentrations about one-third to that of sea water (~300-400 mOsm). Rather than having high intracellular salt levels, they accumulate low molecular weight organic compounds, or "osmolytes", in the intracellular fluid which stabilise cells against osmotic stress and help in maintaining osmotic equilibrium (Yancey et al. 1982). Commonly occurring osmolytes are classified as sugars, polyols, amino acids and their derivatives, methylamines and methylsulfonium compounds (Yancey 2005). Since these organic osmolytes can be accumulated in extremely high concentrations without disturbing cellular functions, they are also called compatible solutes. Methylamines such as TMAO counteract the perturbation of proteins by urea (Yancey and Somero 1980, Treberg et al. 2006) and therefore are also known as counteracting solutes. Some osmolytes are also known to stabilise the cellular components and are referred to as compensatory solutes (Gilles 1997).

The types of osmolytes used by marine taxa vary, and many organisms use a combination of osmolytes. For example, Carr et al. (1997) showed that molluscs use predominately glycine betaine (GB, Figure 1.2) and taurine, crustaceans use the α-amino acid glycine and GB, while creatine, lactate and TMAO were the dominant components in fish. Not only do osmolytes assist in maintaining osmotic equilibrium, but they also have protective and stabilising properties.

1.5.2 Osmolytes as dietary biomarkers

An osmolyte is suitable as a dietary biomarker if: 1) the osmolyte is not made by the predator (not part of mammalian synthesis); 2) after absorption from the gastrointestinal tract is

complete, osmolyte concentrations in circulation fall monotonically; 3) the osmolyte is not stored in the body and there is no release during prolonged fasting (Lydersen et al. 2002), and 4) the osmolyte occurs in prey in sufficient quantities to be detectable in the predator's plasma.

In addition to TMAO and AsB, compounds that meet these criteria are homarine and dimethylsulfoniopropionate (DMSP) (Figure 1.2). TMAO, AsB, homarine and DMSP belong to a group of osmolytes known as *betaines*, which are zwitterionic at physiological pH and are very soluble in water. Betaines contain a fully methylated (positively charged) nitrogen or an analogous tertiary sulfonium group in the case of DMSP. The osmolyte GB does not meet criteria number one because it is synthesised endogenously by both marine animals and mammals; however, a short lived increase in GB blood concentrations has been shown to occur after feeding (Slow et al. 2004, Schwab et al. 2006). Therefore, GB was also tested as a potential dietary biomarker in this thesis. The general properties and functions of TMAO, GB, AsB, DMSP and homarine are discussed below.

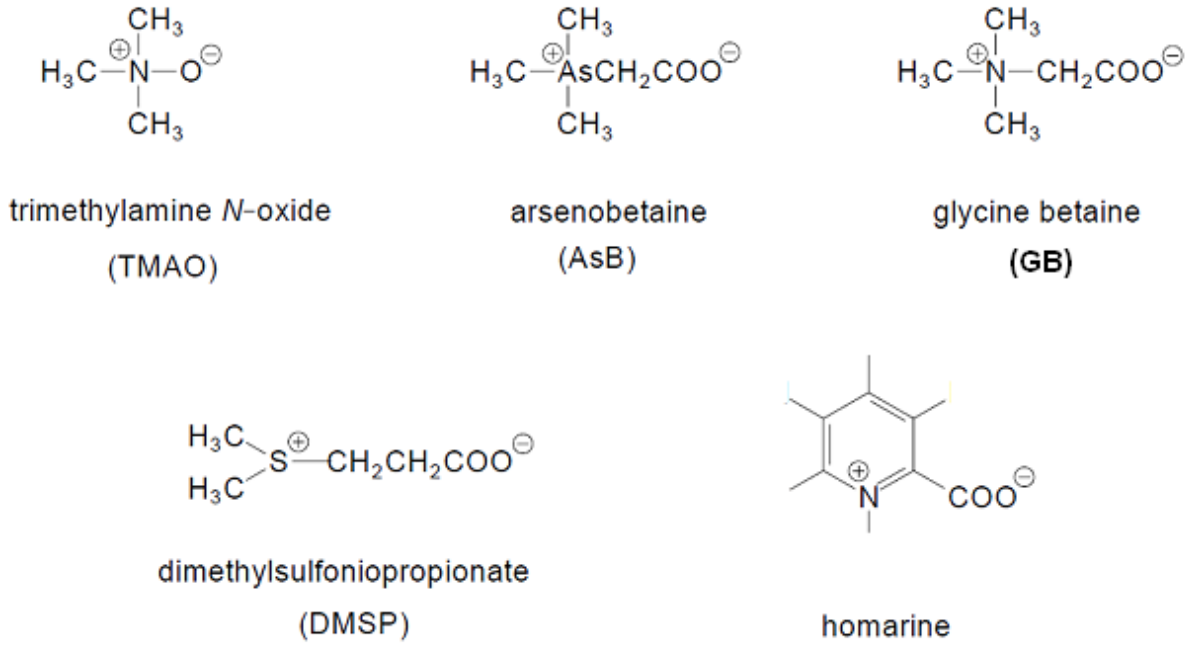


Figure 1.2. Structure of betaines and betaine analogues identified as dietary biomarkers.

1.5.3 Trimethylamine N-oxide (TMAO)

TMAO is a polar, nitrogenous methylamine compound (Figure 1.2) that is present in seawater (Gibb and Hatton 2004) as well as in numerous marine organisms including cartilaginous and teleost fish and invertebrates (Table 1.1). Cartilaginous fish have body tissues that are iso-osmotic to seawater and accumulate large quantities of urea. TMAO acts primarily as an osmoregulatory compound in cartilaginous fishes to counteract the destabilising effects of urea (Yancey and Somero 1980). TMAO may also help reduce the denaturing effects of hydrostatic pressure on protein structure in deep-sea animals. Several studies have demonstrated a correlation between TMAO and depth, showing that organisms living in deeper habitats contain significantly more TMAO than species living in shallower habitats (Gillett et al. 1997, Kelly and Yancey 1999, Treberg et al. 2002). TMAO may also increase buoyancy in cartilaginous fish because it reduces the density of body fluids (Withers et al. 1994a, Withers et al. 1994b). To date, TMAO has been measured in the muscle of seven Antarctic fish species (Table 1.1). TMAO levels in cold-adapted teleosts are significantly greater than those from temperate regions and are upregulated during the winter season, suggesting TMAO may also play a role as a cellular cryoprotectant (antifreeze) by reducing the freezing point of body fluids (Raymond 1998, Raymond and Devries 1998). Amongst Antarctic species that have been studied, the toothfish *Dissostichus mawsoni* has the highest concentrations of TMAO in muscle tissue (153.9 mmol kg^{-1}, Raymond and DeVries 1998). Toothfish are also considered to be an important prey item for Weddell seals (Ainley and Siniff 2009). In cartilaginous fish and cold-adapted teleosts, TMAO is present in high quantities in the muscle, but is found in lower concentrations in blood and organs such as the liver (Raymond and Devries 1998). In general, higher concentrations of TMAO are found in cartilaginous and cold-adapted fish, species living at deeper depths, and in muscle tissue.

In mammals, dietary TMAO ingested after fish consumption is reduced to trimethylamine (TMA, Figure 1.3), a tertiary amine, in the gastrointestinal tract by bacteria. TMA is then absorbed and removed by the liver, where TMA is oxygenated back to TMAO by the microsomal flavin-containing monooxygenase (FMO3). TMAO is then either transported to various tissues as an osmolyte or excreted in the urine. TMAO is also acquired through foods (*e.g.*, legumes, meat and eggs) that are high in choline and carnitine (Figure 1.3) that may be converted to TMA by gut bacteria (Zhang et al. 1999).

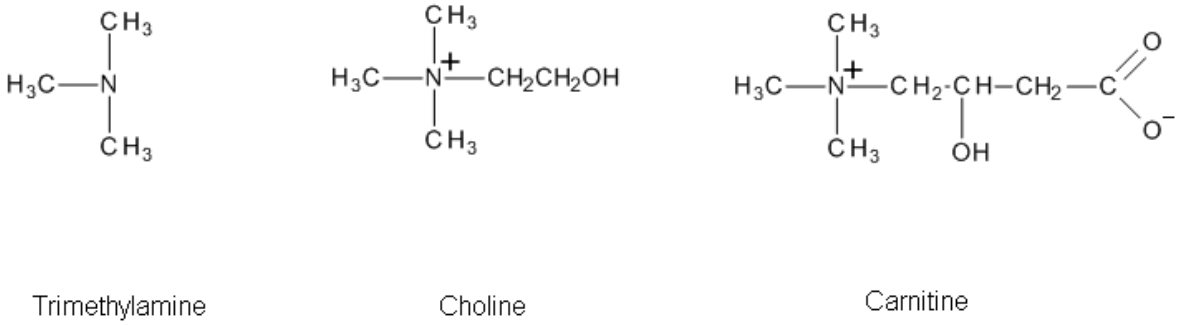


Figure 1.3. Chemical structures of trimethylamine, choline and carnitine.

Based on experiments involving humans and rodents, TMAO appears to be rapidly excreted from the body via the urine within 24 hours (Al-Waiz et al. 1987, Mitchell et al. 1997). Plasma TMAO concentrations in rats peaked at one hour after oral administration of TMA (Smith et al. 1994) and it also appears that TMAO has a similar half life in Weddell seals (Eisert et al. 2005).

TMA is responsible for trimethylaminuria, or "fish odour" syndrome, in humans (Mackay et al. 2011). Trimethylaminuria occurs when a mutation occurs in the FMO3 gene. In affected patients, TMA is not efficiently converted to TMAO in the liver but instead accumulates in the urine, sweat and breath leading to a characteristic smell of rotting fish (Al-Waiz et al. 1987). The bacterial degradation of choline to TMA, as well as the reduction of TMAO to TMA, is also responsible for the characteristic 'fishy" odour in rotting fish.

Table 1.1. Concentration of trimethylamine *N*-oxide (TMAO) in some marine animals on a fresh mass basis.

Species	Common name	Tissue	TMAO [mmol kg^{-1}]	Reference
Crustaceans				
Euphausia superba	Antarctic krill	muscle	30	Carr et al. (1996)
Pandalus danae	shrimp	muscle	76	Kelly and Yancey (1999)
Pandalopsis ampla	shrimp	muscle	203	Kelly and Yancey (1999)
Molluscs				
Octopus dofleini	octopus	muscle	13	Carr et al. (1996)
Loligo opalescens	squid	mantle	48	Kelly and Yancey (1999)
Illex argentinus	squid	mantle	55	Carr et al. (1996)
Clione antarctica	Antarctic pteropod	not given	112	Seibel and Walsh (2002)
Berryteuthis magister	squid	mantle	219	Kelly and Yancey (1999)
Gonatus borealis	squid	mantle	333	Kelly and Yancey (1999)
Cartilaginous fish				
Somniosus microcephalus	Greenland shark	muscle	143–170	Anthoni et al. (1991)
Fugaleus ventralis	shark	muscle	166	Withers et al. (1994b)
Bathraja spinosissima	skate	muscle	215	Kelly and Yancey (1999)
Teleost fish				
Osmerus mordax	smelt	muscle/liver	25/6	Raymond (1998)
Parophrys vetulus	English sole	muscle	41	Samerotte et al. (2007)
Clupea harengus	herring	muscle/liver	48/21	Raymond (1994)
Merluccius productus	Pacific hake	muscle	53	Samerotte et al. (2007)
Coryphaenoides cinereus	cod	muscle	121	Gillet et al. (1997)
Coryphaenoides armatus	cod	muscle	173	Gillet et al. (1997)
Antarctic teleosts				
Notothenia gibberifrons	Antarctic cod	muscle	79[a]	Oehlenschläger (1991)
Champsocephalus gunnari	ice fish	muscle	94[a]	Oehlenschläger (1991)
Notothenia neglecta	Antarctic cod	muscle	97[a]	Oehlenschläger (1991)
Chaenocephalus aceratus	ice fish	muscle	106[a]	Oehlenschläger (1991)
Trematomus bernacchii	emerald rockcod	muscle/liver	115/57	Raymond and DeVries (1998)
Pagothenia borchgrevinki	bald notothen	muscle/liver	145/59	Raymond and DeVries (1998)
Dissostichus mawsoni	Antarctic toothfish	muscle/liver	154/81	Raymond and DeVries (1998)

[a] converted from mg TMAO-nitrogen/100 g^{-1} fresh mass

1.5.4 Glycine betaine (GB)

GB was the first betaine to be discovered when it was isolated from the sap of the sugar beet *Beta vulgaris* in the 1860s (Scheibler 1869). It is a highly water–soluble compound containing a quaternary ammonium group at one end and a carboxyl group at the other end (Figure 1.2), and was named for its structural resemblance to the amino acid glycine. GB (sometimes called simply betaine) has a widespread role in osmoregulation in a variety of animals, plants and bacteria including humans (Yancey et al. 1982). It is abundant in marine invertebrates and cartilaginous fish, with concentrations exceeding 50 mmol kg^{-1} in certain molluscs and crustaceans, but present in much lower concentrations in teleost fish (Table 1.2). In the cartilaginous elephant fish *Callorhinchus milii*, GB is the principal counteracting solute (50–70 mmol kg^{-1} fresh mass (Bedford et al. 1998b)). To date, no studies have looked at GB in Antarctic organisms. Carr et al. (1996) describes the GB and TMAO content of *Euphausia superba* in their study, but lists this species of origin as Norway. It is unclear whether this is actually *E. superba*, or a mislabelled species.

GB is also a major mammalian osmolyte and high levels of GB are found in the inner medulla of the kidney in order to counteract high salt and urea levels (Craig 2004). In humans, GB is both provided by the diet and produced by the oxidation of dietary choline in the liver or kidneys, and plasma levels of GB are tightly regulated in normal humans (Lever and Slow 2010). In humans, there is a short-lived increase in plasma GB concentrations following food consumption (peak = 2 hours, Atkinson et al. 2008) even though GB excretion in urine is minimal in non-diabetic individuals (Atkinson et al. 2009, Lever et al. 2012). Instead, GB is stored in tissues and serves as a methyl donor; GB is demethylated by betaine-homocysteine methyltransferase (BHMT) to form dimethylglycine (DMG) and methionine, lowering homocysteine, which is a known risk factor in heart disease (Lever and Slow 2010).

Table 1.2. Concentration of glycine betaine (GB) in some marine animal tissues on a fresh mass basis.

Species	Common name	Tissue	GB [mmol kg^{-1}]	Reference
Invertebrates				
Lamellibrachia barhami	tubeworm	vestimentum	8.6	Yin et al. (2000)
Riftia pachyptila	"	"	10.5	Yin et al. (2000)
Ridgeia piscesae	"	"	109	Yin et al. (2000)
Crustaceans				
Euphausia superba	krill	muscle	17.5	Carr et al. (1996)
Molluscs				
Not given	mussel	not given	10.6	de Zwart et al. (2003)
Not given	clam	soft tissues	16.3	de Zwart et al. (2003)
Callinectes sapidus	blue crab	muscle	22.2	Carr et al. (1996)
Illex illecebrosus	Squid	mantle	125	Carr et al. (1996)
Mytilus edulis	blue mussel	muscle	158	Carr et al. (1996)
Teleost fish				
Not given	groper	not given	0.08	de Zwart et al. (2003)
Not given	salmon	not given	0.12	de Zwart et al. (2003)
Not given	perch	not given	0.17	de Zwart et al. (2003)
Not given	cod	not given	0.16	de Zwart et al. (2003)
Not given	monkfish	not given	3.3	de Zwart et al. (2003)
Cartilaginous fish				
Callorhinchus milii	elephant fish	liver	57.3	Bedford et al. (1998)
	"	heart	66.1	
	"	muscle	71.1	

1.5.5 Arsenobetaine (AsB)

In fish and crustaceans, arsenic is present mainly as quaternary arsonium compounds such as arsenocholine, AsB, tetramethylarsonium ion, trimethylarsonium oxide (the arsenic analogue of TMAO) and arsenolipids, while arsenosugars are predominant in marine algae (Cullen and Reimer 1989, Edmonds and Francesconi 1993). AsB is the arsenic analogue of GB, with an atom of arsenic replacing the nitrogen in the quaternary ammonium group (Figure 1.2). AsB

was first isolated from the tail muscle of the western rock lobster *Panulirus cygnus* by Edmonds et al. (1977), and since then it has been identified as the major arsenic compound in seafood (Table 1.3 and references therein), often constituting up to 95% of arsenic in fish (Cullen and Reimer 1989). AsB has also been identified as the major arsenic compound in the liver of some marine mammals, sea birds and sea turtles (Table 1.3). Within the Antarctic ecosystem, AsB has been detected in various organisms from McMurdo Sound, including the bald notothen *Trematomus bernacchii* and the sea star *Odontaster validus* (Grotti et al. 2010, Table 1.3).

The origin of AsB is not well understood (Francesconi 2010). While sea water contains trace amounts of arsenic mostly as inorganic arsenate ($H_2AsO_4^-$, Andreae 1978, Francesconi 2010), AsB is not present in seawater and ends up in the marine food chain due to it chemical similarity to phosphate, an essential nutrient for algae and other marine biota (Edmonds and Francesconi 1987). Mammals do not synthesise AsB but acquire it through the diet either as AsB or as arsenocholine ($C_5H_{14}AsO^+$), which is converted in the liver to AsB. AsB is absorbed from the gastrointestinal tract and eliminated unchanged *via* urine but the elimination rate of AsB varies between different species with two distinct phases of excretion (Charbonneau et al. 1978, Vahter et al. 1983, Le et al. 1994). For example, in rats, the majority of an intravenous dose of AsB was excreted within 2–12 hours, while the rest was eliminated over a period of several days (Vahter et al. 1983, Yoshida et al. 2001). In human volunteers, AsB ingested with a fish meal was excreted in two distinct phases, with different half lives of *ca*. 7 hours and 63 hours, respectively (Lehmann et al. 2001). Eisert et al. (2005) calculated the half life of AsB in Weddell seals as approximately two days, with the remaining AsB eliminated over a period of one to two weeks. Due to its structural similarity, AsB enters tissues as a GB analogue (Randall et al. 1996), and this is likely the reason for the observed two-phase excretion. AsB is not a substrate for BHMT (Lee et al. 2004), the enzyme that demthylates GB. This is fortunate because demethylation or other degradation reactions would convert the nontoxic AsB to more toxic forms of arsenic (Khokiattiwong et al. 2001, Jenkins et al. 2003). The fact that AsB is not metabolised in mammals may also account for the relatively long half-life of AsB compared to GB (Eisert et al. 2005).

Table 1.3. Concentration of arsenobetaine (AsB) in seaweed, marine animal and mammal tissues. Concentrations are on a fresh mass basis unless indicated otherwise.

Species	Common name	Tissue	AsB [μmol kg^{-1}]	Reference
Invertebrates				
Sterechinus neumayeri	sea urchin	soft tissues	16.1–64.9[a,b]	Grotti et al. (2010)
Odontaster validus	sea star	soft tissue	56.9–186.7[a,b]	Grotti et al. (2010)
Parborlasia corrugatus	nemertina worm	soft tissues	261[a,b]	Grotti et al. (2010)
Molluscs				
Laternula eliptica	clam	soft tissues	38.9–67.3[a,b]	Grotti et al. (2010)
Antarctic teleosts				
Trematomus bernacchii	bald notothen	muscle	644–908[a]	Grotti et al. (2010)
Pinnipeds				
Pusa hispida	ringed seal	muscle	0.8	Fujihara et al. (2003)
	""	kidney	2.5	Ebisuda et al. (2003)
	""	liver	5.5	Ebisuda et al. (2003)
Erignathus barbatus	bearded seal	liver	2.3	Goessler et al. (1998)
Callorhinus ursinus	northern fur seal	liver	4.0	Fujihara et al. (2003)
Phoca groenlandica	harp seal	liver	13.5[a]	Kubota et al. (2003)
Cetaceans				
Delphinapterus leucas	beluga whale	liver	0.07	Goessler et al. (1998)
Globicephalus melas	pilot whale	liver	6.9	Goessler et al. (1998)
Phocoenoides dalli	Dall's porpoise	liver	8.9[a]	Kubota et al. (2003)
Sea turtles				
Caretta caretta	loggerhead turtle	liver	6.15	Kubota et al. (2003)
Chelonia mydas	green turtle	liver	10.5	Fujihara et al. (2003)
Eretmochelys imbricata	hawksbill turtle	liver	44	Fujihara et al. (2003)
Seabirds				
Diomedea nigripes	black-footed albatross	liver	68	Fujihara et al. (2003)
Larus crassirostris	black-tailed gull	liver	100	Fujihara et al. (2003)
Seaweed				
Undaria pinnatifida			18.8	Choi et al. (2011)
Laminaria japonica			401	Choi et al. (2011)

[a] μmol kg^{-1} dry mass, [b] Antarctic species

1.5.6 Dimethylsulfoniopropionate (DMSP)

DMSP is a tertiary sulfonium analogue of GB (Figure 1.2). DMSP is produced by phytoplankton and certain marine algae (Van Alstyne et al. 2001) during the synthesis of methionine, a sulfur-containing amino acid (Figure 1.4; Gage et al. 1997, Kocsis et al. 1997). DMSP is thought to function as an osmolyte in algae and copepods (Reed 1983, Tang et al. 1999), as a cryoprotectant and antioxidant in algae (Kirst et al. 1991, Karsten et al. 1992, Sunda et al. 2002), as a feeding attractant or foraging cue in coral reef fishes (Nakajima 1996, Debose et al. 2006, Debose et al. 2008) and as a feeding deterrent in some marine plants (Van Alstyne et al. 2001). DMSP has also been shown to stabilise proteins at low temperatures, but it may have the opposite effect and perturb protein function at high temperatures (Nishiguchi and Somero 1992).

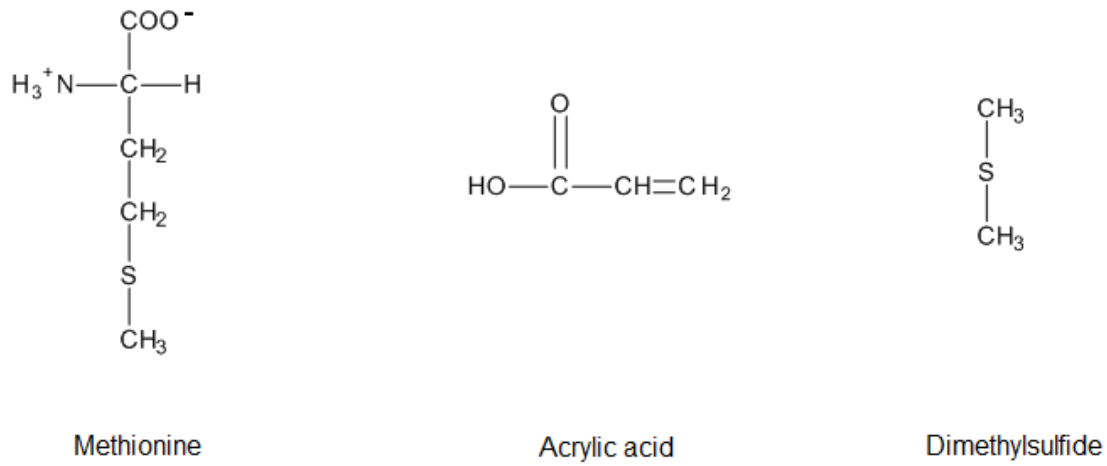


Figure 1.4. Chemical structures of methionine, acrylic acid and dimethylsulfide.

DMSP is present in seawater and has been identified in a number of marine organisms and coral species (Keller et al. 2004, Yancey et al. 2010). However, the presence and concentrations of DMSP compared to TMAO and GB appear to be significantly less, especially in marine fishes (Table 1.4). DMSP is not synthesised endogenously by animals; instead, DMSP is obtained through the diet or through symbiosis (Levasseur et al. 1994, Tang et al. 1999). For example, Atlantic cod in the Northeast Atlantic accumulate DMSP in their tissues after feeding on the pteropod *Limacina helicina*. *Limacina helicina* in turn graze on

phytoplankton that contain high levels of DMSP (Levasseur et al. 1994). The highest concentrations of DMSP in marine animals have been found in the mantle and gills in *Tridacna* species, a group of marine clams from Micronesia (up to 57 mmol kg^{-1}; Hill et al. 2000, Hill et al. 2004). This is due to the clams' symbiotic association with dinoflagellates (zooxanthellae) which synthesise large quantities of DMSP. To date, the distribution of DMSP has not been studied in Antarctic marine organisms. However, DMSP has been detected in *L. helicina* in McMurdo Sound (Elliott et al. 2009). *Limacina helicina* grazes on the phytoplankton *Phaeocystis antarctica* which is responsible for DMSP production in McMurdo Sound during the summer (Rellinger et al. 2009). It is possible that some fish may accumulate DMSP in their tissues after feeding on *L. helicina* in a similar scenario that is observed with cod in the Northeast Atlantic.

While DMSP itself is not volatile, DMSP is degraded by marine bacteria to form acrylic acid and dimethylsulfide (DMS, Figure 1.4), which is a greenhouse gas and an important component of the global sulfur cycle (Andreae 1990). Although DMSP is odourless and tasteless, DMS has a distinctive marine odour often described as an "off" or "seaweed" smell, which is attributed to the enzymatic or thermal breakdown of DMSP in seafood (Brooke et al. 1968, Levasseur et al. 1994). DMS is also responsible for the sulfurous "blackberry feed" smell of Atlantic cod (Levasseur et al. 1994). Acrylic acid is known to deter feeding and it has been suggested that DMSP may therefore be used as a defence mechanism in marine plants (Van Alstyne et al. 2001, Van Alstyne et al. 2007).

Due to its structural similarity to GB, DMSP is metabolised by BHMT (Slow et al. 2004). In rodents, 66% of DMSP was shown to be excreted *via* urine within 24 hours following injection (Slow et al. 2004) while Levasseur and colleagues (1994) found that DMSP had a fast clearance rate in the pteropod *L. helicina*, with up to 65% of DMSP clearing within 12 hours.

Table 1.4. Concentration of dimethylsulfoniopropionate (DMSP) in some marine animals and algae on a fresh mass basis.

Species	Common name	Tissue	DMSP [mmol kg^{-1}]	Reference
Crustaceans				
Acartia tonsa	copepod	whole	0.02[a]	Tang et al. (1999)
Temora longicornis	copepod	whole	1.03[a]	Tang et al. (1999)
Molluscs				
Limacina helicina	Antarctic pteropod	whole	0.05	Elliot et al. (2009)
Mytilus edulus	mussel	whole	0.2-4	Hill et al. (1995)
Geukensia demissa	ribbed mussel	digestive gland	0.12–0.59	White et al. (1995)
		body	0.09–0.15	
Tridacna maxima, T. squamosa	giant clam	adductor	4.7	Hill et al. (2000)
		syphonal mantle	31.2	Hill et al. (2000)
		gill	33.4	Hill et al. (2000)
		byssal mantle	37.4	Hill et al. (2000)
Hippopus hippopus	giant clam	adductor	1.2–9.5	Hill et al. (2004)
		gill	7.0–47.4	Hill et al. (2004)
Tridacna crocea	giant clam	adductor	1.5–3.4	Hill et al. (2004)
Tridacna derasa	giant clam	adductor	1.4–13.9	Hill et al. (2004)
		gill	6.3–8.8	Hill et al. (2004)
Fish				
Sparisomo veride	parrotfish	muscle	0.001	Dacey et al. (1994)
Stegates dorsopunicans	damselfish	muscle	0.008	“’
Acanthurus coeruleus	surgeonfish	muscle	0.048	“”
Sparisomo radians	parrotfish	muscle	0.32	“”
Algae				
Brown algae			0.05–0.32	Dacey et al. (1994)
Red algae			0.13–0.68	“”
Ulva lactuca	green algae	holdfast, blade, tip	37–224	Van Alstyne et al. (2007)
Cnidaria				
Porites lobata	coral		0.19	Yancey et al. (2010)
Pocillopora damicornis	coral		3.18	Yancey et al. (2010)

[a] nmol/individual

1.5.7 Homarine

Homarine is a quaternary ammonium cyclic betaine (Figure 1.2) that was first isolated from the muscle of the lobster, *Homarus* sp. (Hoppe-Seyler 1933). Homarine has since been identified in various tissues of marine invertebrates (Table 1.5 and references therein). Ito et al. (1994) also detected homarine in a number of organs from ten species of fish from Japan. Concentrations varied amongst the type of organ and species of fish, with the highest homarine concentrations found in the gonads of marine fish (up to 22 mmol kg^{-1}). Homarine so far appears to be absent from freshwater species (Leonard and Macdonald 1963, Ito et al. 1994) and there is no data at present on the presence of homarine in Antarctic fishes.

Little is known about the physiological function of homarine and whether it assists in osmotic regulation. Homarine has been shown to act as a feeding deterrent in the Antarctic gastropod *Marseniopsis mollis* that contains high concentrations of homarine (McClintock et al. 1994). The authors found that the predatory sea star *Odontaster validus* rejected individuals of *M. mollis* but fed on small pieces of fish. *Odontaster validus* also rejected filter paper discs treated with homarine (McClintock et al. 1994). Netherton and Gurin (1982) propose that homarine may contribute to the synthesis of TMAO in crustaceans by acting as a transmethylating agent, transferring its *N*-methyl group (CH_3, Figure 1.2) to form not only TMAO, but betaine and choline. During this demethylation process, homarine loses its methyl groups to form picolinic acid, which in turn can be methylated to form homarine (Netherton and Gurin 1982).

Homarine is endogenously synthesised by the pink shrimp *Penaeus durorarum* from GB which donates two carbon atoms and the nitrogen atom in homarine synthesis (Hall and Gurin 1975, Netherton and Gurin 1980). It is not clear whether homarine is synthesised endogenously by all marine invertebrates, or acquired through diet. Homarine is not known to be metabolised by mammals and there are no data at present on its excretion rates by mammals.

Table 1.5. Concentration of homarine in some marine animals. Concentrations are on a fresh mass basis unless indicated otherwise.

Species	Common name	Tissue	Homarine [mmol kg^{-1}]	Reference
Crustaceans				
Callinectes sapidus	blue crab	muscle	2.8	Carr et al. (1996)
Chionoecetes opilio	crab	gill	5.3	Hirano (1975)
Pandalus borealis	shrimp	muscle	5.8	Hirano (1975)
Panaeus japonicus	prawn	muscle	0.8–12.3	Suwetja et al. (1989)
Euphausia superba	Antarctic krill	muscle	7.5–18.2[b]	Hirano (1975)/Carr et al. (1996)
Molluscs				
Haliotis iris	abalone	whole	0.07–0.08	Wells et al. (1998)
Marseniopsis mollis	gastropod	mantle	3.6[b]	McClintock et al. (1994)
	"	foot	21	McClintock et al. (1994)
Crassostrea gigas	oyster	muscle	9.7–10.5	Suwetja et al. (1989)
Octopus vulgaris	octopus	muscle	10.3	Suwetja et al. (1989)
Illex argentinus	squid	mantle	12.5	Carr et al. (1996)
Siliqua patula	razor clam	soft parts	23.7	Carr et al. (1996)
Echinoderms				
Holothuria atra	Black sea cucumber	ovaries	3.9[a]	Bandaranayake and Des Rocher(1999)
		viscera	8.2[a]	""
Hemicentrotus pulcherrimus	sea urchin	muscle	6.9–9.2	Suwetja et al. (1989)
Cnidaria				
Actinia equina	sea anemone	tentacle	10.9	Mathias et al. (1960)
Teleost fish				
Clupea pallasi	Pacific herring	muscle	0.3	Ito et al. (1994)
Argyrosomus argentatus	white croaker	muscle	0.3	""
Argyrosomus argentatus	white croaker	liver	0.6	""
Clupea pallasi	Pacific herring	liver	0.7	""
Sardinops melanosticus	sardine	muscle	0.8	""
Engraulis japonica	anchovy	muscle	0.9	""
Engraulis japonica	anchovy	ovary	4.1	""
Sardinops melanosticus	sardine	ovary	22.4	""

[a] dry mass basis, [b] Antarctic species

1.6 Thesis aims and research hypotheses

The earlier work of Eisert et al. (2005) provided evidence that some female Weddell seals feed during lactation but only through the detection of TMAO and AsB. The opportunity to expand on the biomarker method led to the following main areas of research and hypotheses addressed in this thesis:

1. Many but not all female Weddell seals feed during lactation.

2. If females are feeding their plasma will contain the biomarkers TMAO, AsB, homarine, DMSP and elevated levels of GB.

3. Measuring biomarkers in Weddell seal prey will tell us what the seals are feeding on if it can be shown that different prey types contain characteristic/specific biomarkers.

4. Measuring several different biomarkers reduces the risk of failing to detect food consumption.

In order to examine the diet composition of individual lactating Weddell seals, knowledge of the biomarker distribution within Weddell seal prey was required. It was hypothesised that TMAO, GB, DMSP, AsB and homarine would vary sufficiently between different prey species (Chapter Four) that they could be useful biomarkers for the consumption of particular prey types (Chapter Five). Faecal studies in McMurdo Sound indicate that Weddell seals feed predominantly on Antarctic silverfish with smaller contributions of *Pagothenia borchgrevinki* and *Trematomus* spp. (Castellini et al. 1992, Burns et al. 1998). *Dissostichus mawsoni* is thought to be an important prey item based on observational evidence (Kim et al. 2005, Ponganis and Stockard 2007) but toothfish consumption is rarely detected using traditional diet methods. Data from McMurdo Sound are in contrast to other parts of the Antarctic, such as East Antarctica and the Weddell Sea, where Weddell seals are reported to eat a much more diverse diet including a variety of fish (nototheniids and channichthyids), crustaceans and cephalopods (Green and Burton 1987, Plötz et al. 1991, Lake et al. 2003) and along the Antarctic Peninsula where myctophids and cephalopods contribute significantly to the diet in some years (Casaux et al. 1997, 2006, 2009).

5. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and liquid chromatography tandem-mass spectrometry (LC-MS/MS) assays will be able to detect and measure these biomarkers in seal plasma and prey.

The study by Eisert et al. (2005) employed two different analytical methods for measuring biomarkers. TMAO was measured using high performance liquid chromatography (HPLC) and AsB by graphite furnace atomic absorption spectrometry (GF-AAS) (Eisert et al. 2005). Two of the main aims of this thesis were to develop and validate an ^{1}H NMR assay for extracting, detecting and measuring biomarkers in fish and invertebrate prey (Chapter Two) and an LC-MS/MS assay that could be used to identify and quantify all of the biomarkers in a single plasma sample using one analytical system (Chapter Three). These instruments were chosen based on prior (Slow et al. 2004, Lee et al. 2006) and current (Lenky et al. 2012) methods that have been developed for measuring betaines in the laboratory of Dr. Michael Lever at the Canterbury Health Laboratories in Christchurch, New Zealand.

6. If lactating Weddell seals are feeding, they are selectively feeding on energy-rich prey.

A third component of this thesis examined the gross composition of Weddell seal prey (Chapter Six). If feeding is an essential part of the lactation strategy for some females (Chapter Five), then energy content of prey is a key factor for estimating the food requirements and energy flux from mothers to pups and for determining reproductive success of Weddell seals. This information is important in its own right for understanding a species' ecological role in the food web based on its nutritional value, distribution, and biology and for understanding how certain resources or environmental conditions may restrict breeding colonies to areas of local prey abundance. If some females need to forage, it would mean they would be limited to places where there is adequate prey and suitable access to that prey. On the other hand, if females are not foraging during lactation, they would be much less affected by changing environmental conditions that affect food resources and their physical environment.

Chapter Seven is a synthesis of the main results of the thesis. It refers back to the research aims and hypotheses and directions for future research are discussed. References are included in one section at the end of the thesis.

Publications resulting from this thesis thus far:

Lenky, C.C., McEntyre, C.J., Lever, M. (2012) Measurement of marine osmolytes in mammalian serum by liquid chromatography-tandem mass spectrometry. Analytical Biochemistry 420:7-12.

Lenky, C., Eisert, R., Oftedal, O., Metcalf, V. (2012) Proximate composition and energy density of nototheniid and myctophid fish in McMurdo Sound and the Ross Sea, Antarctica. Polar Biology 35(5):717-724.

Chapter Two

Development and validation of a method for measuring dietary biomarkers in marine organisms using ^{1}H NMR spectroscopy

500 MHz NMR instrument used in this research. Photo by C. Lenky.

2.1 Introduction

2.1.1 Nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) is a widely used technique for identifying compounds in tissues (Lin et al. 2007, Wu et al. 2008), urine (Maschke et al. 1997, Bollard et al. 2001, Slow et al. 2004) and plasma (Samuelsson et al. 2006, Tiziani et al. 2008). NMR provides a profile of the metabolites present in the body fluids or tissues, which is then used to characterise an organism's physiological state. NMR has a wide range of applications in the biological sciences, and has been applied extensively in toxicology, food science and environmental studies (Warne et al. 2001, Viant et al. 2003, Martinez et al. 2005).

NMR is a standard tool used by organic chemists for determining the structure of unknown compounds. During ^{1}H NMR experiments, hydrogen nuclei present on compounds in a sample are subjected to a strong magnetic field. Depending on the molecular structure of compounds, hydrogens will resonate at varying frequencies and signals (peaks) produced by proton resonance are observed at different positions along the NMR spectrum. This position, termed the chemical shift (δ), allows for identification of individual analytes. Additionally, the signal produced is directly proportional to the number of hydrogens present. For example, the protons of a methyl group (CH_3) produce a signal three times the intensity of the intensity produced by a single-carbon-bound proton (CH) (Lambert and Mazzola 2004). ^{1}H NMR is attractive to biologists because it can be used to identify and quantify multiple compounds within a complex mixture simultaneously. The advantages of using NMR are that it requires minimal sample preparation, is non-destructive (so samples can be measured repeatedly), and is able to provide a large amount of data relatively quickly (~5 minutes per sample). Some disadvantages are that it is a relatively insensitive technique (detection limits of 10 µM are acceptable) and requires large sample sizes (~500 µL) (Fan et al. 1993, Fan 1996, Wishart 2008). In aqueous solutions a large water signal dominates the ^{1}H NMR spectrum around 4.7 ppm which can swamp any nearby peaks of interest. However, presaturation techniques that scramble the magnetism of water protons (giving them no signal) can be utilised (Lambert and Mazzola 2004).

To prepare samples for ^{1}H NMR, tissues (*e.g.*, liver or muscle) are typically flash-frozen in liquid nitrogen (N_2) and kept frozen at -80 °C or lyophilised to prevent enzymatic activity and

maintain concentrations of metabolites (Wishart 2008). In order to disrupt cells, tissues are normally ground in a mortar and pestle cooled with liquid N_2. However, this method is labour-intensive and considerable care is required when transferring frozen powder to minimise sample loss. Once the cells are disrupted, acids or organic solvents are used to extract different metabolites. A wide range of extraction solvents have been utilised by various groups including perchloric acid (Chang et al. 1995, Le Belle et al. 2002, Martinez et al. 2005, Lin et al. 2007) or mixtures of acetonitrile/water (Griffin et al. 2001, Lin et al. 2007), methanol/water (Lin et al. 2007), and methanol/chloroform/water (Le Belle et al. 2002, Wu et al. 2008). Perchloric acid (PCA; $HClO_4$) is commonly used to precipitate proteins but it can also give rise to broad resonances on the NMR spectra. PCA is often neutralised to a pH of 7.0–7.4 with potassium carbonate (K_2CO_3) in order to remove perchlorate ions (Fan 1996). The potassium perchlorate precipitate is removed by centrifugation, and the polar supernatant is lyophilised and re-suspended in a sodium phosphate buffer (Viant 2003). In NMR metabolomics studies, both Lin et al. (2007) and Wu et al. (2008) found that methanol/chloroform/water extractions produced the highest metabolite yield and reproducibility in fish tissues. One advantage of this method is that it extracts hydrophilic and hydrophobic components into different fractions. The disadvantages are that it is time consuming, and requires multiple solvent additions, mixing and centrifugation steps. Like the PCA-based methods, extracts from procedures using methanol to separate polar and non-polar fractions are often also re-suspended in a neutral buffer (Viant 2007, Wishart 2008). While these extractions allow for the analysis of hundreds of compounds in a single sample, all of the methods listed above require multiple steps and dilutions and there is likely to be loss of analyte.

Extracts are combined with deuterium oxide (D_2O) and an internal standard (Lambert and Mazzola 2004). D_2O is used as an internal lock for locking the magnetic field at the deuterium frequency, which compensates for any drifting. Otherwise, peaks would appear at different frequencies over time. For quantitative work, the choice of internal standard is important for obtaining reliable results. An ideal internal standard should be stable in the sample matrix and during preparation, and not be volatile. Factors which must also be considered are: 1) whether the internal standard is sufficiently soluble in the sample matrix; 2) whether it binds to proteins or other components of the sample; and 3) the resonance of the internal standard does not overlap with the resonance of other compounds on the NMR

spectrum likely to be present in the sample. The peak height of the internal standard is used to normalise peak heights of other compounds and quantify unknown samples. Some internal standards that have been used in quantitative NMR studies include: sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (TSP or TMSP), acetonitrile, *tert*-butanol and hexadeutero-4,4-dimethyl-4-silapentane-1 ammonium trifluoroacetate (DSA) (Lee et al. 2006, Lin et al. 2007). Pivalamide (2,2-dimethylpropanamide) was chosen as the internal standard in this study because it is inexpensive, is a non-volatile solid, dissolves well in the sample matrix, and has nine identical protons similar to TMAO, GB and AsB and thus produces a strong signal. To my knowledge this is the first study to use pivalamide as an internal standard.

2.1.2 Application of ^{1}H NMR to studying dietary biomarkers

One advantage for using ^{1}H NMR to measure TMAO, GB, AsB, DMSP and homarine is that the methyl groups present on these compounds all give rise to a single peak in the spectrum, making quantification straightforward by measuring the peak height of the signal (Figure 2.1). TMAO, GB and AsB each have nine identical methyl protons, DMSP has six and homarine three (Figure 2.1). A number of studies have measured these biomarkers in marine animals, yet few have done so using ^{1}H NMR methodologies. For example, AsB is often measured as a component of total arsenic by inductively coupled plasma–mass spectrometry (ICP-MS, Amlund et al. 2006) or GF-AAS (Eisert et al. 2005). DMSP is normally converted to DMS and analysed by gas chromatography (Hill et al. 2000, Van Alstyne et al. 2006) while TMAO is reduced to TMA and measured using the the ferrous sulphate/EDTA method of Wekell and Barnett (1991) (*e.g.*, Raymond 1998, Raymond and DeVries 1998, Kelley and Yancey 1999, Treberg and Driedzic 2002). TMAO and GB have so far been measured in tissues of elasmobranch fish and deep-sea animals (Bedford et al. 1998a,b; Yin et al. 2000) and homarine in abalone and marine mussels using ^{1}H NMR (Viant et al. 2003, Rosenblum et al. 2005, Jones et al. 2008, Tuffnail et al. 2009) while AsB and DMSP have been measured using ^{1}H NMR, but in rat liver (Lee et al. 2004).

This chapter describes a new ^{1}H NMR method for identifying and quantifying TMAO, GB, AsB, homarine and DMSP in a variety of marine species collected from the Ross Sea and McMurdo Sound regions in Antarctica. The method was first tested using New Zealand seafood purchased from a local seafood market. The method was then applied to selected

Antarctic samples in order to compare biomarker concentrations with those published in the literature.

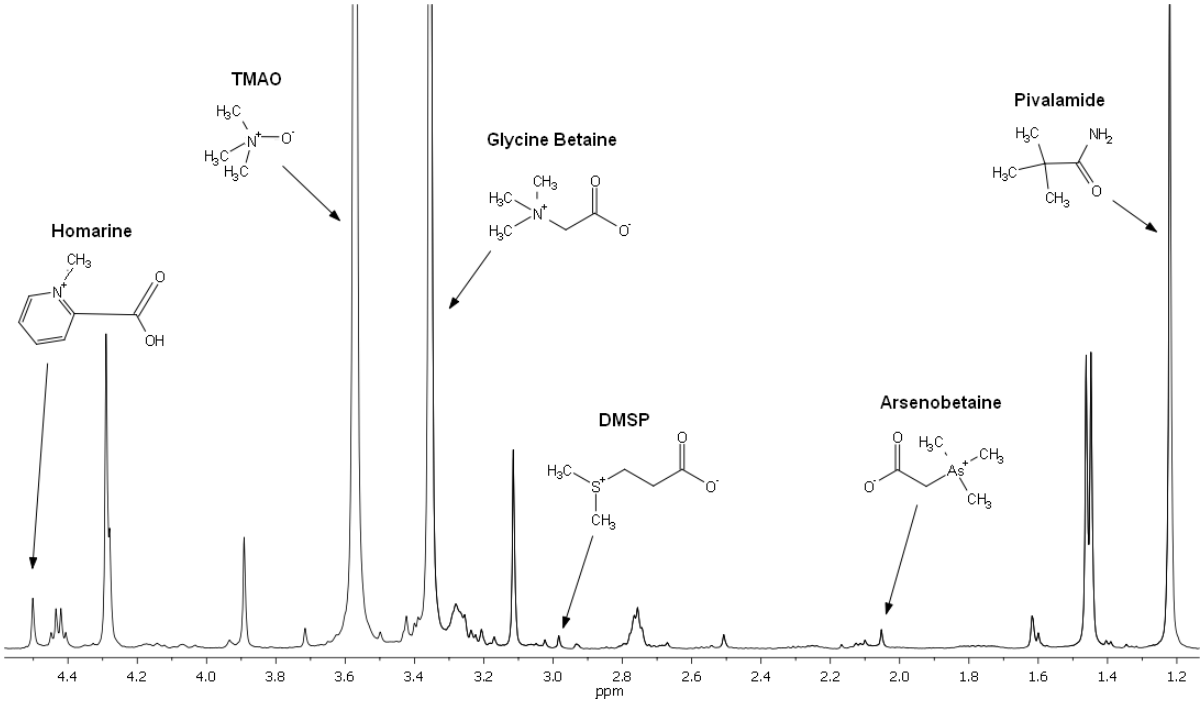


Figure 2.1. ^{1}H NMR spectrum showing the positions of the measured singlets for homarine (4.51 ppm), TMAO (3.53 ppm), glycine betaine (3.35 ppm), DMSP (2.98 ppm) and arsenobetaine (2.05 ppm) in relation to the internal standard pivalamide (1.22 ppm) in aqueous standard. Note the convention is that chemical shift (δ) is displayed right to left.

2.2 Methods

2.2.1 Materials

TMAO, GB, AsB and pivalamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMSP was previously synthesised in the lab of Dr. Michael Lever (Canterbury Health Laboratories) by the method of Samuelsson et al. (1998) and homarine from picolinic acid after the method of Cornforth and Henry (1952). Analytical grade PCA was obtained from APS Specialty Chemicals (NSW, Australia), 99.9% D_2O from Global Science and Technology Ltd (Auckland, New Zealand) and analytical grade dichloromethane (DCM) from Mallinckrodt Chemicals (Paris, KY, USA).

2.2.2 Sample preparation

Sea perch (Sebastidae: *Helicolenus barathri*), arrow squid (Ommastrephidae: *Notodarus sloanii*) and green mussel (Mytilidae: *Perna canaliculus*) were purchased fresh from a local seafood market in Christchurch, New Zealand. Whole sea perch and squid (n = 1 each) were cut into small pieces, then distilled water was added to the sample at a 1:1 ratio and the mixture was homogenised in a common kitchen food blender (Kitchen Aid) to form a smooth homogenate. Mussel shells (n = 3) were opened by cutting the posterior and anterior adductor muscles, and the foot muscle was removed. Mussel tissues were then pooled and ground in distilled water using an Ultra Turrax homogeniser (model IKA T25)[3]. One gram each of sea perch, squid or mussel homogenate was then weighed into a 10 mL Corning tube and extracted using 3 mL of cold 6% PCA (to precipitate proteins) and 1 mL DCM to separate polar and non-polar (lipid) fractions. Samples were vortexed for 30 seconds and then shaken at room temperature for 30 minutes. The polar and lipid layers were separated by centrifugation at 12,000*g* for 5 minutes. The polar supernatant was then transferred into clean, labelled 10 mL centrifuge tubes. This extraction procedure was performed three times on the one gram of homogenate. Subsequent extracts were pooled in the same tube, and the total volume of supernatant was recorded.

Preliminary validation experiments were carried out to ensure adequate recovery of analytes using this extraction protocol: a one gram subsample of sea perch was extracted five times using 3 mL aliquots of PCA, with successive extracts (1 to 5) treated as separate samples. The extractions (1 to 5) were then prepared for NMR as described in section 2.2.3. As shown in Figure 2.2, 90% of total extractable TMAO is recovered after one extraction, while 100% is recovered after three extractions.

The extraction method described above was then applied to whole Antarctic fish and fish tissues in order to: 1) detect and quantify biomarkers and 2) compare TMAO concentrations in white muscle and liver with those published by Raymond and DeVries (1998). *Trematomus bernacchii*, *T. nicolai* and *T. pennellii* were collected by hook and line. Immediately after capture, fish were anaesthetised with 0.2 g L^{-1} tricaine methanesulfonate (MS-222), euthanized by spinal cord dislocation and snap frozen whole using liquid N_2. A

[3] The blender was not used to homogenise mussel tissue due to the small amount of sample

900-gram white muscle fillet of *Dissostichus mawsoni* was provided by Dr. Art DeVries (University of Illinois, Urbana, USA). In the laboratory, fish were allowed to partially thaw, cut into small pieces and homogenised after adding water at a 1:1 ratio as above. Separate samples of *Pagothenia borchgrevinki*, *T. bernacchii* and *Gymnodraco acuticeps* were also collected by hook and line. Liver and white muscle was excised from these fish after euthanasia, wrapped in foil and snap frozen in liquid N_2. One-gram subsamples of whole fish, white muscle or liver homogenate were extracted with PCA and processed as described above.

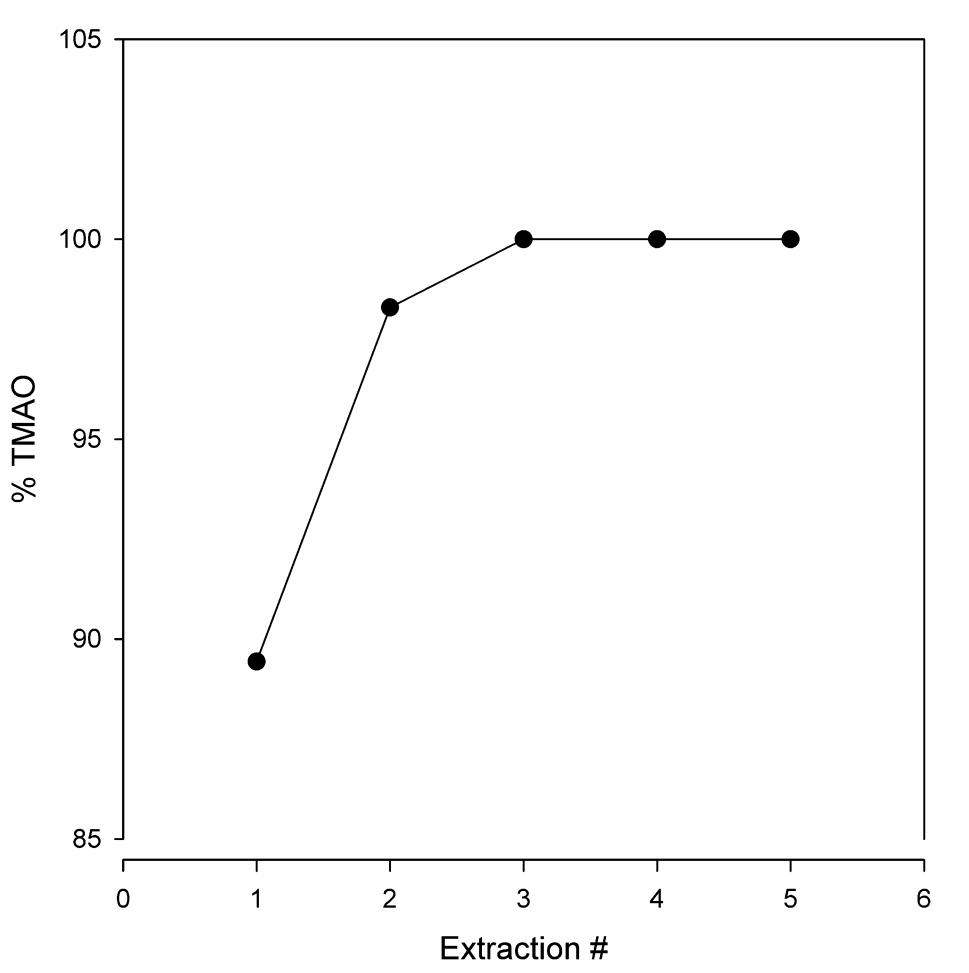


Figure 2.2. Fish (sea perch) homogenate extracted five times using 3 mL aliquots of 6% perchloric acid. 90% of total extractable TMAO was recovered in the first extraction and 100% of TMAO was recovered by the third extraction.

2.2.3 ^{1}H NMR analysis and spectral processing

A 400 µL subsample of extract (pH = ~1) was added to 200 µL of D_2O containing 5 mM pivalamide as the internal standard. All spectra were measured on a 500 MHz Varian INOVA instrument at 23 °C using 5 mm NMR tubes. Spectra were obtained using an 8.1 µs (90°) pulse with a 2.0 second relaxation delay. Spectral width was set to 6000 Hz. Thirty-two scans were collected for each spectrum into 64k data points. A water-suppression technique (PRESAT) was used to remove the large water signal using the following settings: presaturation delay = 3.5 s, satpwr = 25. Spectra were zero-filled to 128k points with an exponential line broadening of 1.0 Hz before Fourier transformation.

Spectral processing was carried out using MestReNova software, version 6.0.4-5850 (www.mestrec.com, Mestrelab research, Santiago de Compostela, Spain). All spectra were referenced to the resonance of pivalamide (1.22 ppm) and peak heights of each biomarker were measured after manual phasing and baseline correction. In order to quantify the biomarkers, external standards containing known concentrations of analytes were included in each run. Aqueous standards of GB and TMAO were prepared from 1 to 150 mmol L^{-1}. Because of differences in biological concentrations, AsB and DMSP were prepared at 10 to 1,000 µmol L^{-1} and homarine from 1 to 30 mmol L^{-1}. Calibration standards were prepared by independent dilution from either a 10 mM or 1 M stock standard. Calibration curves were constructed by plotting standard concentration *versus* the peak height ratio of the analyte relative to pivalamide (Figure 2.3). The linearity of the method was determined by linear regression analysis. The peak height ratios of analyte:internal standard were then used to quantify biomarkers in the prey (unknown) samples. The final concentrations of biomarkers in tissues/whole prey were calculated after accounting for the dilution of the sample.

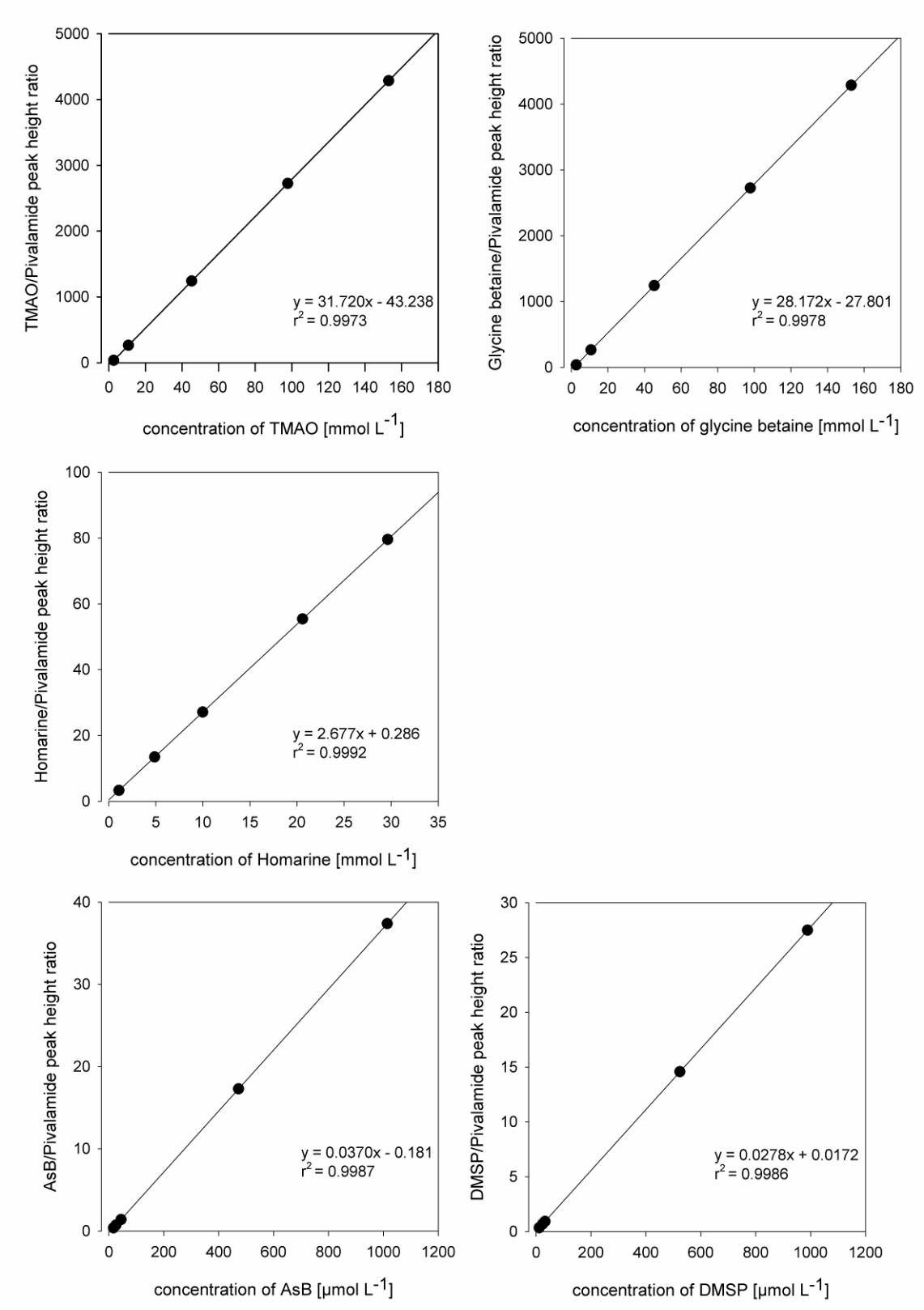


Figure 2.3. Standard curves for TMAO, glycine betaine, homarine, AsB and DMSP measured by ^{1}H NMR.

2.2.4 Precision and recovery

TMAO, GB, AsB, DMSP and homarine were added to chicken breast homogenate to obtain 'low' and 'high' quality control (QC) materials similar to the range of concentrations found in marine fish samples. These QC samples were used to determine recovery and analytical precision. A piece of chicken breast fillet (purchased from the local supermarket) was used because it was expected to contain only GB but none of the other biomarkers of interest. The fillet was cut into small pieces and homogenised with a 1:1 ratio of water using an Ultra Turrax to form a watery paste. Low and high levels of the biomarkers from a 10 mM stock solution (independent of the stock solution used to prepare calibration standards) were then added to subsamples of chicken homogenates (1 g) and extracted as described in section 2.2.2. The low QC contained an added level of 100 µmol kg^{-1} AsB and DMSP, 5 mmol kg^{-1} homarine, 10 mmol kg^{-1} GB and 25 mmol kg^{-1} TMAO. The high QC contained an added level of 500 µmol kg^{-1} AsB and DMSP, 15 mmol kg^{-1} homarine, 50 mmol kg^{-1} GB and 100 mmol kg^{-1} TMAO.

Five batches of four replicates (n = 20) of the low and high spiked QC's were analysed. GB, TMAO, AsB, DMSP, and homarine were measured in the replicates. The within batch and between batch coefficients of variation (CVs) were calculated for each compound. The recoveries of added analytes were measured to provide an indication of the accuracy of the method because standard reference material is only available for AsB (DORM-3, National Research Council of Canada). Because there is a reasonable expectation for chicken breast to be free of marine biomarkers, recovery of added biomarkers except GB should be a fair indication to what extent the method determines the true content of biomarkers in tissue. For this assay to be acceptable, the precision and accuracy at each QC level was required to be within 20% of the true value. Recovery for each analyte was calculated using the following equation:

$$\frac{\text{average result High QC- average result low QC}}{\text{difference in added concentration}} \times 100$$

2.2.5 Limit of detection (LOD) and limit of quantitation (LOQ)

Sensitivity was evaluated by determination of the limit of detection (LOD) and the limit of quantitation (LOQ). The LOD is the lowest concentration of analyte in a sample that can be detected but not quantified, *i.e.*, the concentration of analyte that produces a signal that can be distinguished from the background noise with a certain degree of confidence. The LOQ is the lowest amount of analyte in a sample that can be measured with acceptable accuracy and precision (Rosing et al. 2000). A number of different methods have been proposed for determining the LOD or LOQ but there is no consensus as to which method is the most appropriate, primarily because assays differ in the way that blank values are determined (Gautschi et al. 1993, Mocak et al. 1997, Boqué et al. 2000, Rosing et al. 2000, Linnet and Kondratovich 2004, Wilson et al. 2004, Armbruster and Pry 2008, Welz et al. 2008, Kirsch et al. 2010). For this study, the LOD and LOQ were calculated using two of the methods described in the literature. First, the baseline or method detection limit (LOD_b) for ^1H NMR was determined by measuring the signal-to-noise ratio (S/N = 3) for low concentrations of analytes in aqueous standards (Rosing et al. 2000). The idea behind this method is that an analyte peak is not a "true" peak unless its reaches three times the standard deviation of the noise on the baseline. To calculate the LOD_b, spectral x-y data were exported as an ASCII (.txt) file and imported into a scientific graphics program (SigmaPlot, Systat Software, version 11.0). To determine the deviation of the noise on the baseline, the height deviation from the average value was measured, and the standard deviation calculated in a region where no analyte peaks occurred (Figure 2.4). The LOD_b was then calculated using the peak height and known concentration of each analyte in the same spectrum according to:

$$\frac{\text{concentration of analyte added} * (3 * \text{SD of the noise})}{\text{peak height of analyte}}$$

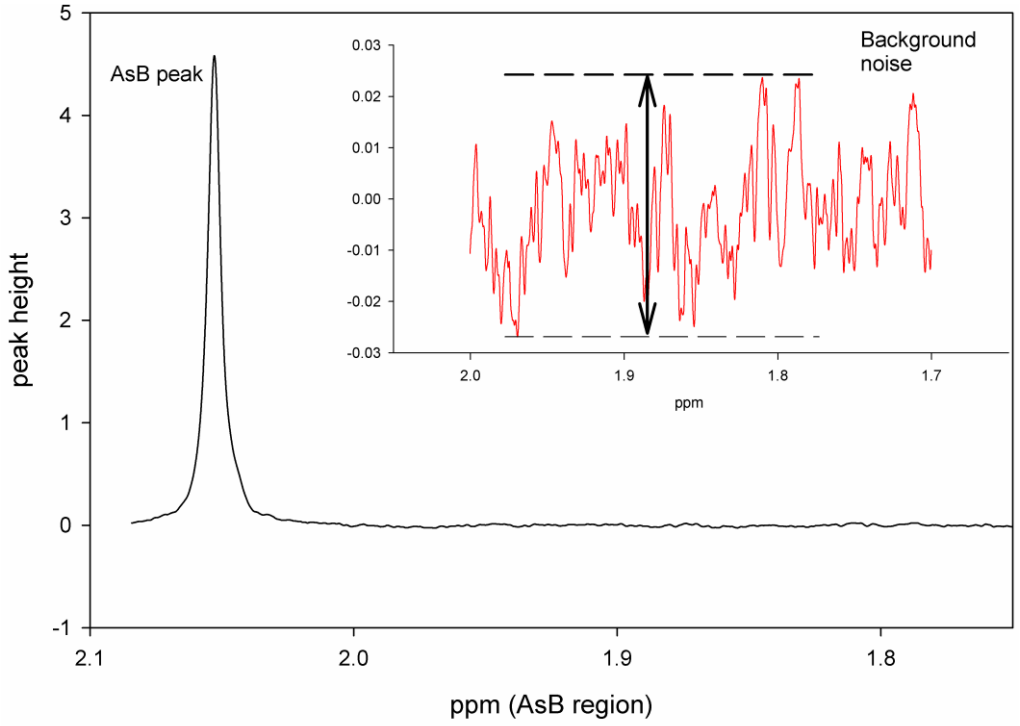


Figure 2.4. Region near 2.05 ppm used in calculating signal-to-noise ratio for AsB.

Calculation of the LOD using this method is valid for matrix-free solutions but not for sample matrices (*e.g.* fish extract). LOD_b in a matrix-free solution represents the absolute lower limit of detection of the instrument. Baseline noise, interference from endogenous substances and other factors affecting signal response may differ between a standard solution and unknown sample. Therefore, sample limits of detection (LOD_s) and quantitation (LOQ_s) were also determined. The LOD_s and LOQ_s were calculated from precision profiles using the relationship between biomarker concentrations in Antarctic samples and precision of the CV of analytical replicates, defined as the relative standard deviation (RSD) by Welz et al. (2008). As the concentration of an analyte decreases, the precision or RSD, increases. The RSD was determined for the replicates (n = 3) of each sample as:

$$\mathrm{RSD} = \frac{s}{\bar{x}} \times 100$$

where s is the standard deviation and x is the mean of the replicate measurements. By using rational or exponential equations to fit a curve through raw data points, it is possible to

extrapolate back to a RSD to find the LOD_s and LOQ_s (Figure 2.5). The LOD_s was set at 20% RSD and LOQ_s at 15% RSD. DMSP is shown as an example (Figure 2.5).

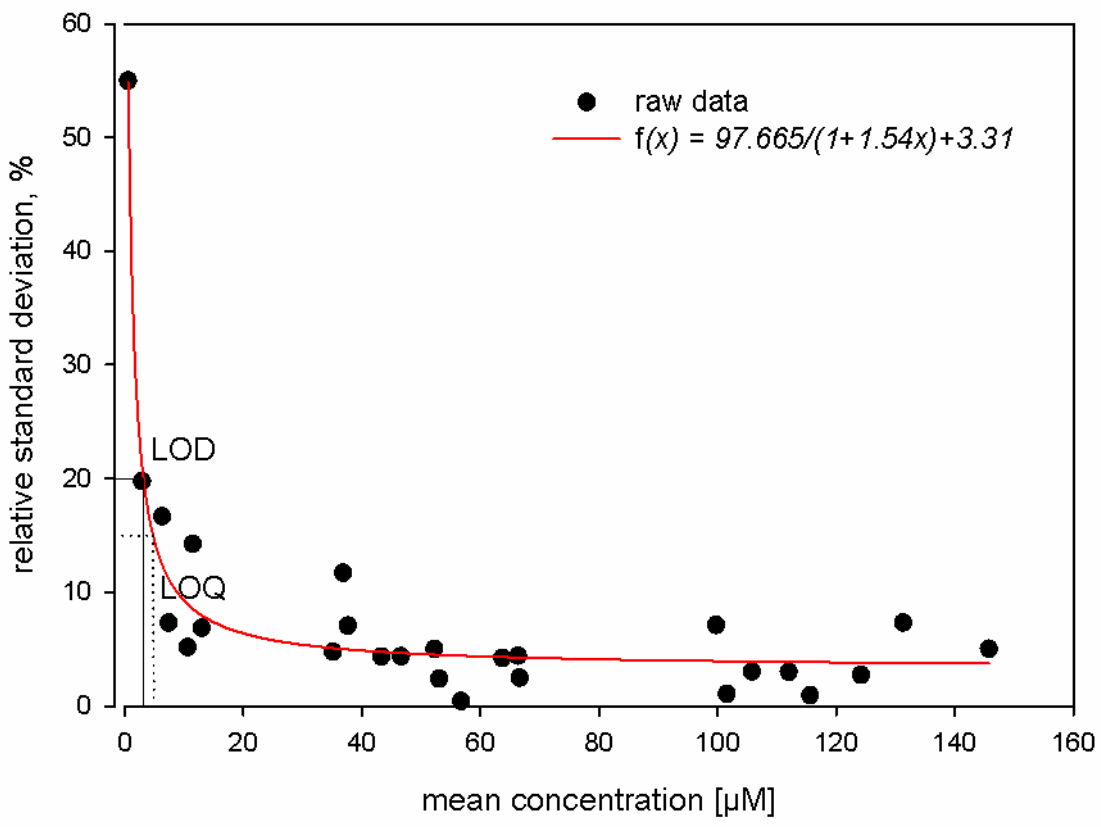


Figure 2.5. Precision *versus* concentration for DMSP in Antarctic prey species.

2.3 Results

2.3.1 Biomarker identification and quantification

The large water peak and the fact that TMAO was present in much larger concentrations in some samples compared to the other analytes led to distortions in the spectral baseline. This was corrected by smoothing the baseline using the "Bernstein polynomial" and manual phasing functions in MestreNova. For some samples, AsB, DMSP and homarine could not be accurately identified or quantified using MestreNova. In this case, spectral x-y data were exported as ASCII files and imported into SigmaPlot to permit plotting of spectral data and the use of curve-fitting algorithms to describe peaks. Deconvolution was carried out by

iteratively fitting the data to sums of Lorentzian peaks, using the Marquardt-Levenberg algorithm (SigmaPlot 11.0).

In test samples, AsB was detected in all species but only after importing spectra into Sigma Plot and applying the deconvolution procedure described above. DMSP was detected only in green mussel (Figure 2.6B). TMAO was the predominant peak in arrow squid and sea perch (Figure 2.6A,C) but not in mussel. Homarine was detected in arrow squid and green mussel (Figure 2.6A,B) but not in sea perch. GB was present in all three species.

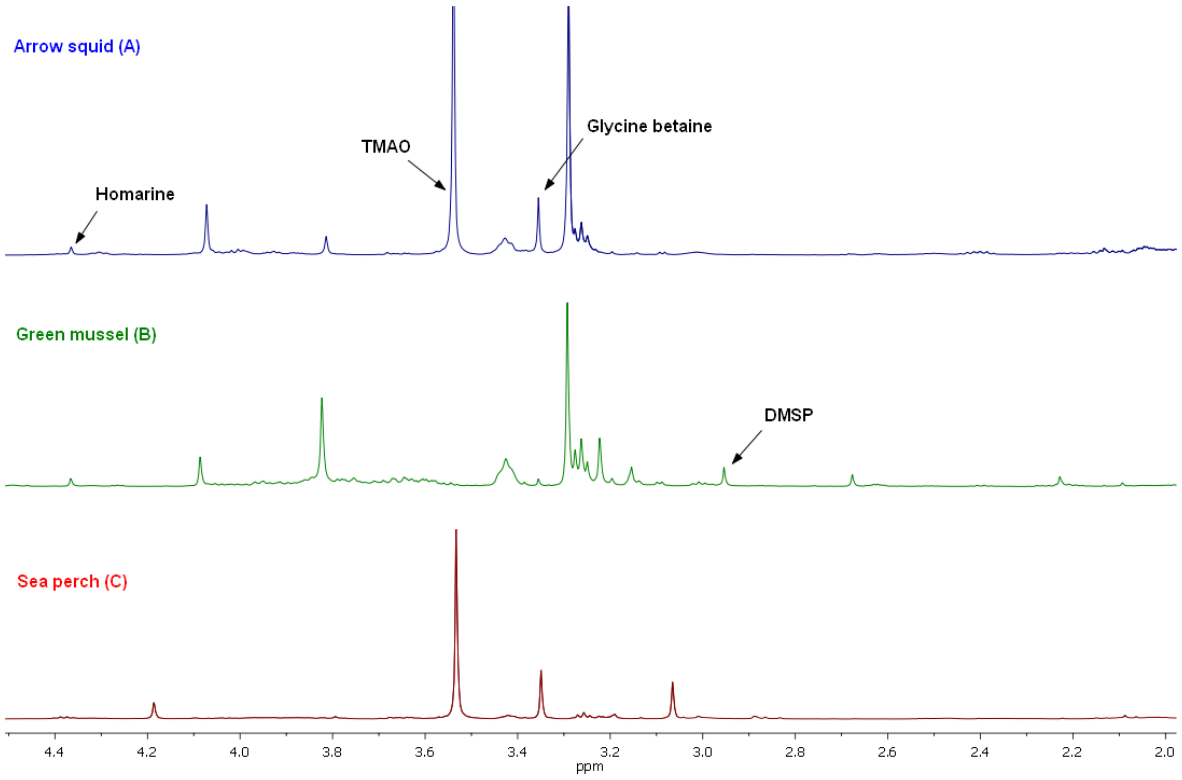


Figure 2.6. ^{1}H NMR spectra for perchloric acid extracts of arrow squid (A), green mussel (B) and sea perch (C).

For whole Antarctic fish, TMAO, GB, DMSP and AsB were detected in *T. bernacchii* and *T. pennellii* (Figure 2.7A,B) whereas only TMAO, GB and AsB were detected in *T. nicolai* (Figure 2.7C). Homarine was not detected by NMR in any of these fish. TMAO was the dominant peak in all spectra.

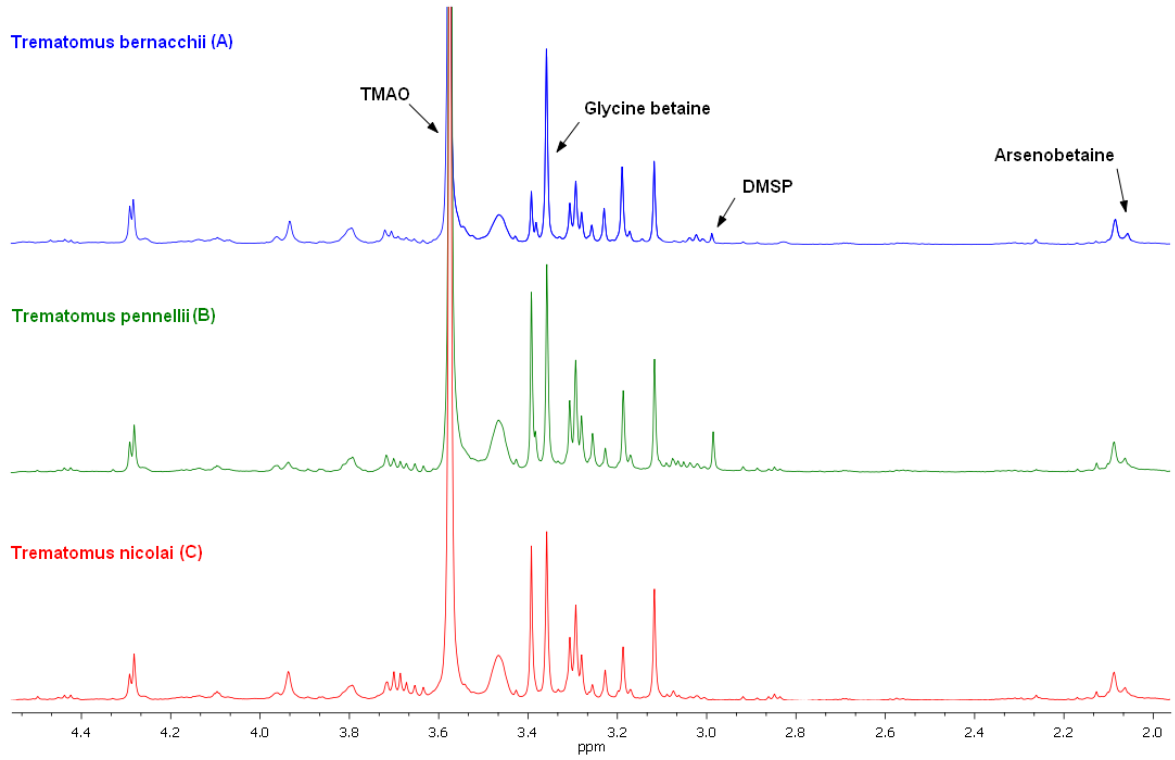


Figure 2.7. ^{1}H NMR spectra for perchloric acid extracts of whole Antarctic fish. *Trematomus bernacchii* (A), *T. pennellii* (B) and *T. nicolai* (C).

For fish tissues (muscle and liver), TMAO was identified in all white muscle and liver samples (Figures 2.8 and 2.9, Table 2.1). GB and AsB were also detected in the liver of *P. borchgrevinki*, *T. bernacchii* and *G. acuticeps* (Figure 2.8). DMSP was detected in *P. borchgrevinki* and *T. bernacchii* liver (Figure 2.8A,B) but not in liver of *G. acuticeps*. TMAO was the dominant peak in all white muscle extracts (Figure 2.9). GB and AsB were also identified in all extracts; however, DMSP was only detected in white muscle of *T. bernacchii* and *P. borchgrevinki* and not *D. mawsoni*. Homarine was not detected in white muscle or liver from any of the species. There was good agreement between concentrations of TMAO in liver and white muscle obtained in this study with those found by Raymond and DeVries (Table 2.1) who used the ferrous sulfate-EDTA method of Wekell and Barnett (1991) to analyse TMAO. Only the TMAO values for *T. bernacchii* were outside the standard deviations obtained by Raymond and DeVries. TMAO concentrations in liver were approximately half that of muscle concentrations on a fresh-tissue basis for all species, which agree with the results of Raymond and DeVries (Table 2.1).

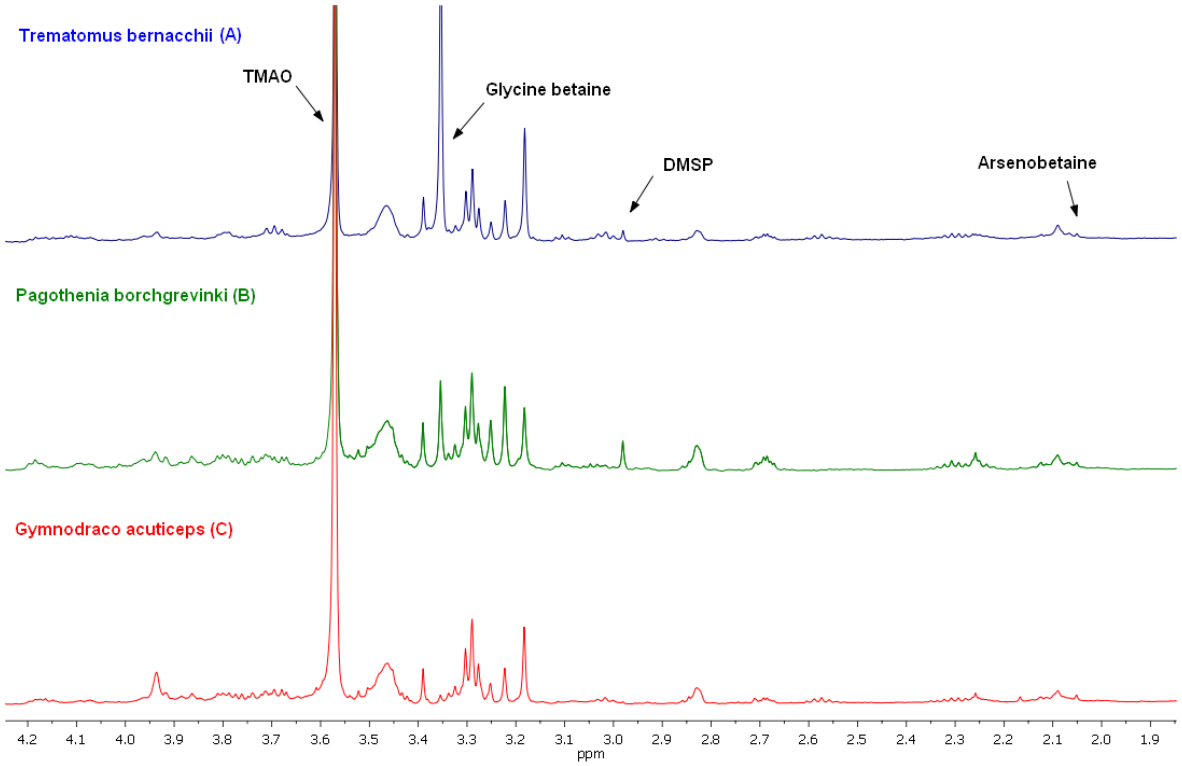


Figure 2.8. ^{1}H NMR spectra for perchloric acid extracts of liver tissue. *Trematomus bernacchii* (A), *Pagothenia borchgrevinki* (B) and *Gymnodraco acuticeps* (C).

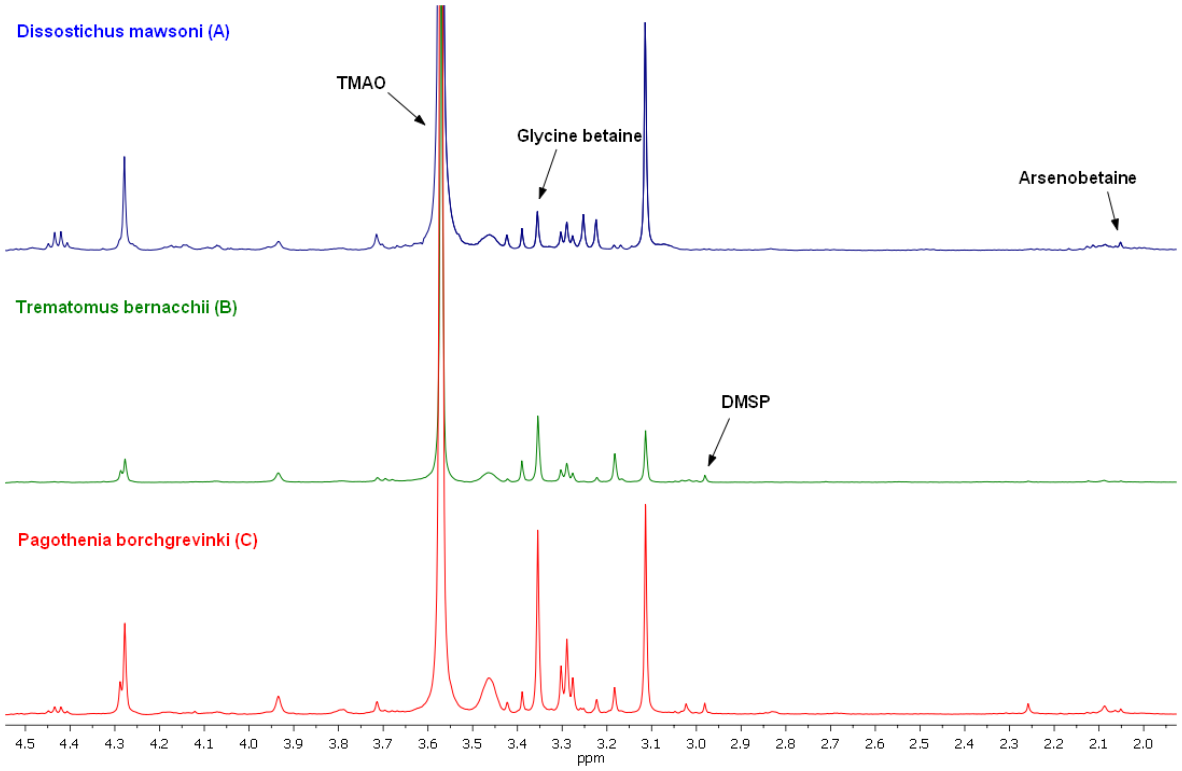


Figure 2.9. ^{1}H NMR spectra for perchloric acid extracts of Antarctic fish white muscle. *Dissostichus mawsoni* (A), *Trematomus bernacchii* (B) and *Pagothenia borchgrevinki* (C)

Table 2.1. Comparison of TMAO concentrations (mmol kg^{-1} wet weight, ± SD) in white muscle and liver of some fish from McMurdo Sound, Antarctica with reported values. Number of fish given in parentheses. N/A = tissue not available.

Species	Muscle	Liver	Reference
Dissostichus mawsoni	153.9 ± 20.4 (3) 162.1 (1)	81.0 ± 2.7 (5) N/A	Raymond and DeVries 1998 this study
Gymnodraco acuticeps	147.5 ± 8.6 (3) NA	84.1 ± 6.7 (3) 72.5 (1)	Raymond and DeVries 1998 this study
Pagothenia borchgrevinki	144.7 ± 13.8 (6) 141.3 (1)	59.4 ± 11.0 (5) 48.8 (1)	Raymond and DeVries 1998 this study
Trematomus bernacchii	115.1 ± 16.1 (4) 87.1 ± 0.6 (3)	56.6 ± 0.7 (3) 33.8 ± 5.7 (4)	Raymond and DeVries 1998 this study

TMAO, GB, AsB and DMSP were detected in both white muscle and whole fish samples of *T. pennellii* and *T. bernacchii* (Table 2.2). Except for DMSP in *T. bernacchii*, concentrations of the biomarkers were greater in white muscle than when the entire fish was analysed (Table 2.2).

Table 2.2. Comparison of TMAO, GB, DMSP and AsB concentrations (mmol kg^{-1} wet weight, ± SD) in white muscle and whole fish of *Trematomus pennellii* and *T. bernacchii* collected from McMurdo Sound, Antarctica in 2007.

Species	White Muscle	Whole fish	Analyte
Trematomus pennellii	124.8 ± 20.3 (2)	72 (2)[a]	TMAO
	20.4 ± 6.6	9	GB
	0.8 ± 0.3	0.01	DMSP
	0.4 ± 0.03	0.1	AsB
Trematomus bernacchii	87.1 ± 0.6 (3)	68.6 ± 2.9 (4)	TMAO
	16.5 ± 7.0	9.4 ± 5.8	GB
	0.4 ± 0.2	0.5 ± 0.1	DMSP
	0.1± 0.04	0.1 ± 0.1	AsB

[a] data is from only two fish pooled together, so no SD was calculated

2.3.2 Precision and recovery

The results of the precision and recovery study for each biomarker in spiked chicken breast homogenate are shown in Table 8. The within-batch CV ranged from 0.6-7.2, and from 0.9-5.1 for the between batch CV (Table 2.3). The analytical recovery of biomarkers for a tissue matrix was between 83 and 107%.

Table 2.3. Results of precision and recovery study of low and high added levels of biomarkers in chicken muscle quality control samples.

Analyte	Analyte[a] added	*n*	Mean[a]	Within batch CV	Between batch CV	Recovery (%)
GB						
Low	10	20	12	1.9	0.9	
High	50	20	46	0.6	3.3	83
TMAO						
Low	25	20	27	1.8	1.8	
High	100	20	99	0.8	1.7	98
Homarine						
Low	5	20	8.0	7.2	3.6	
High	15	20	18	3.7	5.1	107
AsB						
Low	100	20	87	5.1	3.3	
High	500	20	424	1.9	3.7	84
DMSP						
Low	100	20	80	5.7	7.6	
High	500	20	432	3.0	6.2	88

[a] Concentrations in mmol kg^{-1} for GB, TMAO and homarine and in µmol kg^{-1} for AsB and DMSP.

2.3.3 LOD and LOQ

The method detection limit, LOD_b, using the S/N approach and the LOD_s and LOQ_s derived from replicate precision profiles from Antarctic prey are provided in Table 2.4. The LOD_b values for TMAO and GB in aqueous standards using ^{1}H NMR are similar to those calculated by Lee et al. (2006) in urinary samples (15 µM TMAO and GB, respectively) using the same NMR instrument and the S/N approach. Precison profiles could not be created to calculate the LOD_s and LOQ_s for homarine and TMAO because there were not enough data points to create a precision profile for homarine, and all Antarctic prey contained levels of TMAO above the LOD_b (Figure 2.10). The estimated LOD_s for AsB, DMSP and GB in fish matrix was lower than the LOD_b in aqueous standards suggesting that the S/N approach gave a conservative estimate of the detection limit. Due to the structural similiarity of TMAO to GB,

the LOD_s and LOQ_s for TMAO in prey matrix is likely to be around the same values obtained for GB.

Table 2.4. Limits of detection (LOD) and quantitation (LOQ) in of biomarkers measured in aqueous standards (µmol L^{-1}, LOD_b) and in Antarctic prey matrix (µmol kg^{-1}, LOD_s, LOQ_s) using ^1H NMR. N/C = not calculable.

Analyte	**LOD_b (in aqueous solvent)**	**LOD_s (20% RSD in prey)**	**LOQ_s (15% RSD in prey)**
TMAO	9	N/C	N/C
Glycine betaine	9	0.03	0.07
AsB	8	2.1	3.5
Homarine	118	N/C	N/C
DMSP	10	3.2	4.8

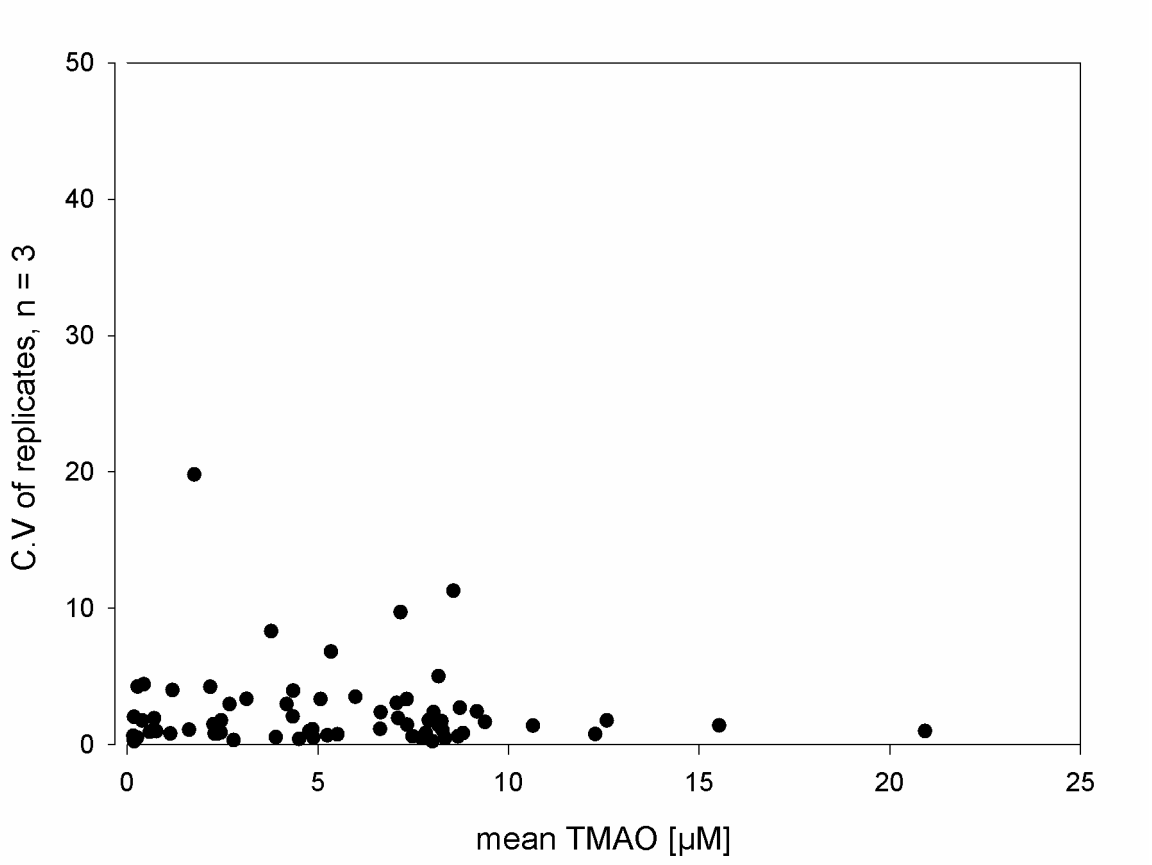


Figure 2.10. Precision *versus* concentration for TMAO in Antarctic prey. As TMAO occurred in relatively high concentrations in all prey analysed, it was not possible to construct a precision profile in the same manner as for the other biomarkers.

2.4 Discussion

The method described here is simple. ^{1}H NMR was able to simultaneously detect TMAO and GB with good peak separation in a five-minute run time. However, it was more difficult to detect AsB, homarine and DMSP because these biomarkers are present at lower concentrations in biological samples than TMAO and GB and the sensitivity of the NMR instrument that was used was insufficient. There was variation in biomarker content and concentration of individual compounds among different prey samples. This is to be expected since osmolyte content may vary both intra- and interspecifically (Carr et al. 1996). Because all analytes of interest are highly water-soluble, samples were initially extracted with water followed by steps to remove contaminants. Perchloric acid was added to precipitate proteins followed by the addition of dichloromethane to remove lipids from the extract. There was

good reproducibility among the samples. Lin et al. (2007) found inconsistencies in peak positions when extracting with perchloric acid, but this problem was not observed in the present study. Although recoveries were low for some compounds (Table 8; GB 83% and AsB 84%), the CV's for this method were good, and TMAO concentrations in tissues from fish from McMurdo Sound were similar to levels found in previous studies (Table 5).

It was difficult to determine the concentrations of AsB, homarine and DMSP from the raw NMR spectrum. In the case of DMSP and homarine, this may be attributed to the fact that both compounds produce inherently weaker signals. DMSP has six methyl protons and homarine three rather than nine methyl protons like TMAO and GB. The resonance of homarine (4.51 ppm) is also close to the chemical shift of water (4.7 ppm). The homarine peak lies in a disturbed region of the water-suppressed spectrum which may contribute to the high LOD_b (118 µmol kg^{-1}) calculated for homarine on NMR. Lee et al. (2006) observed this problem when measuring trigonelline (an isomer of homarine) which has a chemical shift of 4.30 ppm. AsB has nine methyl protons and is expected to produce a strong signal on ^1H NMR, but AsB is present in relatively low concentrations in marine biota. Although AsB is usually the dominant form of arsenic in most marine animals, it is typically present in much lower concentrations than TMAO or GB. The reason for this is that AsB is probably not a 'real' osmolyte but an osmolyte analogue, and is probably only accumulated due to its occurrence in the food chain and structural similarity to 'true' osmolytes (Amlund and Berntssen 2004). AsB is present in *T. bernacchii* from McMurdo Sound at 0.22 mmol kg^1 in white muscle (calculated from Grotti et al. 2010) compared to 115.1 mmol kg^{-1} TMAO (Raymond and Devries 1998) and therefore its quantification by NMR may not be possible for some samples. There was some signal overlap in the region of AsB in some samples, but this was resolved by applying the deconvolution procedure. However, this procedure is time-consuming and the accuracy of the peak heights may not be reliable.

Biomarker concentrations were compared in white muscle and whole fish because for the large Antarctic toothfish *D. mawsoni*, only white muscle was available for analyses (Chapters Four and Six). Results showed that TMAO, GB and AsB concentrations were higher in white muscle than whole fish for *T. pennellii* and *T. bernacchii* (Table 6). However, given that *D. mawsoni* can reach up to two meters in length, there are practical challenges in homogenising a whole toothfish and this was not possible for this study. Additionally, since Weddell seals primarily consume the white muscle of toothfish (Ainley and Siniff 2009) the biomarkers

concentrations detected in a fillet of white muscle for *D. mawsoni* is relevant to actual consumption by seals, in contrast to silverfish and various nototheniid species that are ingested whole by seals and hence were analysed as a whole fish.

It was essential to lower the pH of the fish extracts to pH ~1.0 to correctly separate the methyl resonances of TMAO and GB in the ^{1}H NMR spectrum (Bedford et al. 1998a, Lee et al. 2006). Failure to do so can lead to misidentification of TMAO as GB (Bell et al. 1991, Martinez et al. 2005). In ^{1}H NMR, the chemical shift of a metabolite is affected by the chemical environment. For example, in a neutral buffer solution (pH = 7.5) GB had a chemical shift of 3.22 ppm, AsB 1.89 ppm and DMSP 2.87 ppm (Lee et al. 2004) which were further upfield than chemical shifts determined in this study. Acidifying the samples separates the peaks so that TMAO is further downfield of GB. This was necessary not only because the aim was to quantify both biomarkers but also because TMAO is present in high concentrations in Antarctic fish and may obscure the GB signal (Raymond and DeVries 1998).

Prior to selecting pivalamide, TSP, acetonitrile and *tert*-butanol were also tested as internal standards. However, TSP gave inconsistent results, probably as a result of its low solubility in acidified samples. TSP is a weak acid (pKa ~ 4.5) and at low pH, it is present mainly as a protonated carboxylic acid. When PCA extracts were added to D_2O containing TSP, the mixture turned opaque, indicating that TSP was coming out of solution. This resulted in the TSP peak splitting on the spectrum and, more importantly, random variation in the size of the internal standard peak. This caused inaccurate quantification of analyte concentrations because these are quantified as the ratio of peak heights of analyte: internal standard. Acetonitrile was not suitable as an internal standard because it has a resonance at 2.00 ppm (1.9 ppm, Lee et al. 2006) which is in the region of the AsB signal (2.05 ppm). Acetonitrile also has only one methyl group, so gives a lower signal response than analytes such as TMAO and GB. It is preferable to use an internal standard with a robust signal, again because peak height of the internal standard is used to normalise peak heights of all analytes. *Tert*-butanol is highly soluble in water and has a resonance at 1.20 ppm. This does not interfere with any compounds of interest, but it was inconvenient to work with because it is a solid at room temperature and required melting prior to pipetting. Pivalamide was a convenient internal standard because its resonance does not interfere with the biomarkers being measured and it is highly soluble in the sample matrix.

One disadvantage of ^{1}H NMR is that its sensitivity is inherently low compared with other analytical techniques. By extracting the samples multiple times (*i.e.*, into a relatively large final volume), good recovery was achieved (Figure 2.2) but biomarkers that are normally found in lower concentrations in fish (for example, DMSP) may have been diluted to the point where they could no longer be detected accurately by NMR. Greater analytical sensitivity may be achieved by (a) modifying sample preparation to include steps to pre-concentrate the analyte such as freeze-drying extracts and (b) by modifying NMR parameters, for example by increasing the number of transients collected when obtaining the NMR spectrum. However, this would require longer acquisition times (Lambert and Mazzola 2004). A second option is to use instruments with greater magnetic strength (*e.g.*, 900MHz) or cryogenically cooled probes that can increase spectral resolution and improve detection limits.

^{1}H NMR proved to be an ideal technique to identify TMAO and GB within different matrices (*i.e.*, fish and invertebrates) with minimal sample preparation. NMR is particularly valuable for acquiring profiles on samples where prior knowledge is limited, which will be useful for identifying additional dietary biomarkers in future studies. Additionally, NMR is non-destructive which means samples can be measured repeatedly. However, the NMR instrument used in this study did not have an autosampler, and manually placing samples in the NMR spectrometer and analysing samples in triplicate was time-consuming. Systems with an autosampler would be best suited for this type of quantitative work (Bailey and Marshall 2005).

Due to the potential uncertainty in detecting and quantifying AsB, DMSP and homarine in Antarctic prey samples by NMR and given that these biomarkers were also being measured in the blood plasma from Weddell seals (where they are likely to be found in much lower concentrations than in the prey), other analytical methodologies were considered. The following chapter describes an LC-MS/MS assay for the measurement of biomarkers in seafood and body fluids.

Chapter 3

Development and validation of a method for detecting dietary biomarkers in serum and tissues using liquid chromatography – tandem mass spectrometry

LC-MS/MS instrument used in this study. Photo by C. Lenky

3.1 Introduction

3.1.1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Over the last 10–15 years, LC-MS/MS has become the "gold standard" in clinical laboratories for detecting a number of different analytes associated with drug screening and toxicology, vitamins and related metabolites, steroid hormones and metabolite markers of inborn errors of metabolism (Grebe and Singh 2011). The advantages of LC-MS/MS are that it offers greater analytical specificity than HPLC and higher sample throughput than gas chromatography-mass spectrometry (GC-MS). Sample preparation is also much simpler since LC-MS/MS does not require pre-column derivitization, the process of transforming a compound into a similar compound, or derivative. For analysis by GC-MS, compounds need to be volatile at the temperature needed for separation and also thermally stable (Hübschmann 2009). Derivitization is carried out by a number of processes such as silylation, acylation or akylation depending on the individual properties of the compounds to be analysed. Derivitization increases sampling time and less analytes can be analysed in a single run (Hübschmann 2009). However, in some cases GC-MS is a more sensitive technique. This is due to the fact that GC and MS are compatible because analytes need to be in the gas phase in order to be analysed by mass spectrometry.

The main hurdle in the development of LC-MS was converting the analyte in the mobile phase to gas phase ions in order for them to be analysed by the MS (El-Aneed et al. 2009). This resulted in the need for an interface linking the two techniques. This interface, known as atmospheric pressure ionisation (API), allows for the liquid to be changed into the gas phase and also ionises the analyte. There are a number of different ionisation techniques that may be used with LC-MS. The most common ones used are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) (Pitt 2009). ESI involves *nebulisation*, the process of breaking down a liquid into small droplets. ESI works best over a broader range of different analytes and compounds with higher polarity and molecular weights (including betaines) can be analysed by ESI (Pitt 2009).

Chromatography is used to separate the analytes of interest from each other and from interfering matrix components (Jemal 2000). A sample is injected via an injection port (*e.g.*, autosampler) in the mobile, liquid phase and forced by a high-pressure pump through a

stainless steel column packed with surface-modified silica or polymer particles (stationary phase) where the separation takes place (Kirkland et al. 2010). Reverse-phase chromatography (RPC) is the most commonly used form of liquid chromatography, primarily due to the wide range of analytes that can dissolve in the mobile phase. RPC consists of a non-polar column in combination with a mixture of water plus organic solvents (methanol, acetonitrile, or mixtures of these solvents) as the polar mobile phase. When a sample is passed through the column, non-polar molecules will stick to the column while polar compounds will pass through.

The mass spectrometer measures the mass-charge ratio (*m/z*) of charged particles. There are several types of mass analysers including quadrupole, time-of-flight and ion trap (Pitt 2009). The quadrupole is the most widely used analyser due to the mass range covered and quality of mass spectra for quantitative data (Pitt 2009). Before introduction into the mass spectrometer, analyte molecules are converted from a liquid to gas phase ions. The eluent from the LC system is sprayed through a metal capillary into the ESI source region (Figure 3.1). It is nebulised in a fine mist of droplets by a gas stream and a high temperature. The droplets are electrically charged at their surface where a capillary voltage is applied (Cole 2010). Solvent is evaporated from the droplets using dry nitrogen. At this point, the droplet-clusters divide into smaller droplets (Kebarle and Tang 1993, Kebarle and Verkerk 2009). Sample ions are generated out of the cluster ions and are electrostatically propelled into the vacuum of the mass analyser (Figure 3.1).

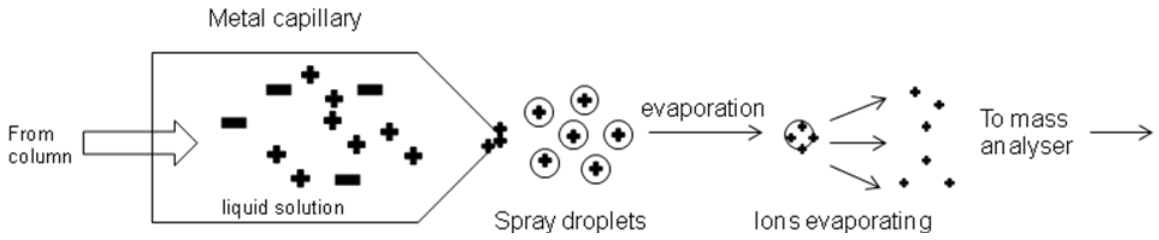


Figure 3.1. Diagram of an electrospray ionisation source (ESI).

A quadrupole mass analyser consists of four parallel rods (Figure 3.2). Ions are passed through the middle of the rods and their motion is influenced by the electric field so that only

ions within a given *m/z* range pass through and are detected (Pitt 2009). In triple quadrupole (tandem) mass spectrometry (which was used in this study), the Q1 and Q3 quadrupoles serve as mass filters, while the Q2 quadrupole functions as a collision cell where fragmentation by collisions with nitrogen or argon gas occurs (Figure 3.2). The ions passed through Q1 are the parent ions, which are molecules of the substance being measured with (most commonly) an additional proton (giving a positive ion: "positive ion mode") or one less proton (giving a negative ion: "negative ion mode"). The ions resulted from the fragmentation are called daughter ions. Selecting particular parent/daughter ion pairs to monitor will increase the sensitivity; this process is called multiple reaction monitoring (MRM) (Grebe and Singh 2011). The main advantage of this technique is that there is a decreased probability of interference from other compounds that have the same or very close molecular weights and product fragments as the analyte of interest. For example, if there are two different molecules that co-elute, they will also need to have the same mass transition (*i.e.*, produce the same fragmented daughter ions) to interfere.

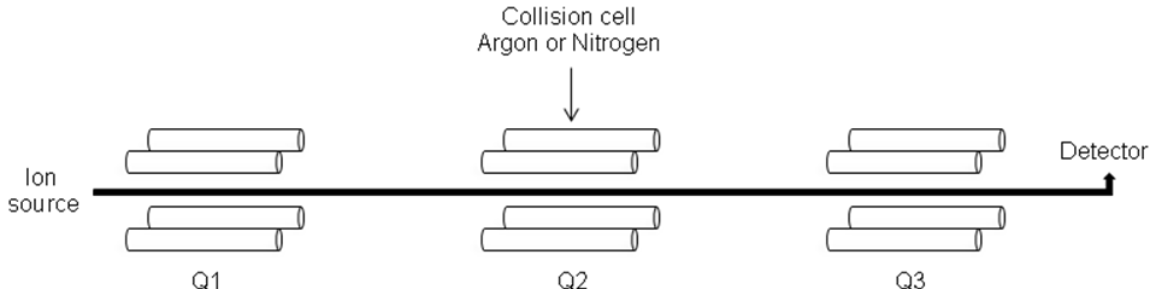


Figure 3.2. A triple quadrupole (tandem) mass spectrometer.

Ion suppression and ion enhancement are two well-known phenomena in LC-MS/MS that affect the precision, sensitivity, and accuracy of an analytical procedure. Components that appear at the same or nearly the same *m/z* value as the analyte of interest can cause spectral interference. These components are primarily made up of endogenous substances present in the sample matrix and in the final extract; while other causes may be exogenous substances, *i.e.*, molecules not present in the sample but may enter from various external sources (reagents, contaminants) during the sample preparation (Jemal 2000). Interference from matrix components can be decreased by injecting smaller sample volumes or by applying more selective extraction techniques during sample preparation. The use of a stable isotope-labelled (*e.g.*, ^{2}H, ^{13}C) internal standard can also counteract these problems, as the internal

standard can be used to normalise the analyte signal (Jemal 2000). The matrix effect on the analyte and on the internal standard is expected to be identical so the internal standard will correct for any errors associated with sample preparation and ion suppression. The deuterated (^{2}H-labelled) internal standard is similar in chemical structure and also has a retention time as close to the analyte as possible. This should effectively reduce the signal variability observed for the analyte and consequently improve the repeatability of the measurement. Unfortunately, not every stable isotope version of an analyte is available and the costs of custom synthesis are often very expensive (Pitt 2009).

3.1.2 Application of LC-MS/MS to measuring dietary biomarkers

TMAO and AsB in the blood plasma of the Antarctic Weddell seal were previously measured separately using HPLC (TMAO) and GF-AAS (AsB) (Eisert et al. 2005). TMAO was found in concentrations ranging from 66 µmol L^{-1} in early lactation to 685 µmol L^{-1} in late lactation, while AsB ranged in concentrations from 0.07 to 0.76 µmol L^{-1}, respectively. GB, TMAO and DMSP have been measured previously in human urine/plasma and coral tissues using LC-MS/MS (Kirsch et al. 2010, Lee et al. 2010, Li et al. 2010) but at present no studies have measured AsB or homarine by LC-MS/MS. One aim of this study was to develop a method that could be used to identify and quantify all of the biomarkers of interest in a single blood sample using one analytical system. This chapter describes a LC-MS/MS method for the simultaneous measurement of GB, TMAO, AsB, DMSP and homarine in serum based on the method of Holm et al. (2003) for measuring GB, choline and DMG.

In Chapter Two, biomarkers were measured in locally purchased seafood and Antarctic prey using ^{1}H NMR. While this method was sufficient for detecting and measuring TMAO and GB, a more sensitive assay than ^{1}H NMR was needed for detecting AsB, homarine and DMSP in Antarctic prey. Initially, a method based on de Zwart et al. (2003) for measuring betaines in various foods was utilised. Validation studies were carried out on sea perch, arrow squid and green mussels, the same species that were used for ^{1}H NMR validation (Chapter Two). However, when the LC-MS/MS method was applied to Antarctic samples low recoveries were measured for all analytes, especially TMAO. Several parameters were tested in order to find the optimal method for measuring these biomarkers in Antarctic prey samples.

3.2 Materials

3.2.1 Reagents

Glycine betaine (HCl salt), TMAO and AsB were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMSP was previously synthesised in the laboratory of Dr. Michael Lever by the method of Samuelsson et al. (1998) and homarine from picolinic acid after the method of Cornforth and Henry (1952). D_9-glycine betaine HCl (D_9-betaine) was obtained from Isotec (Miamisburg, Ohio, USA) and trimethylamine *N*-oxide-D_9 (D_9-TMAO) and $^{13}C_2$-arsenobetaine ($^{13}C_2$-AsB) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Ammonium formate and formic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). HPLC grade acetonitrile (ACN) was obtained from Mallinckrodt (Paris, KY, USA) and HPLC grade methanol (MeOH) was obtained from Merck (Darmstadt, Germany). Bovine calf serum was obtained from ICP Biologicals, Auckland, New Zealand. Sea perch, green mussels, arrow squid and salmon (Salmonidae: *Salmo salar*) were purchased from a local seafood market in Christchurch. Bovine serum was stored at -80 ºC prior to analyses.

3.2.2 Internal standard synthesis

D_6-DMSP and D_4-homarine were not commercially available so they required custom synthesis. D_6-DMSP was synthesised by reacting 3 grams of D_6-dimethylsulfide with 2 grams of acrylic acid in 30 mL of DCM (Figure 3.3). Hydrogen chloride (HCl) gas was produced by dripping sulfuric acid (H_2SO_4) onto sodium chloride (NaCl) and the HCl bubbled through the reaction mixture. D_6-DMSP precipitated out of the solution as a white solid, and was filtered and dried.

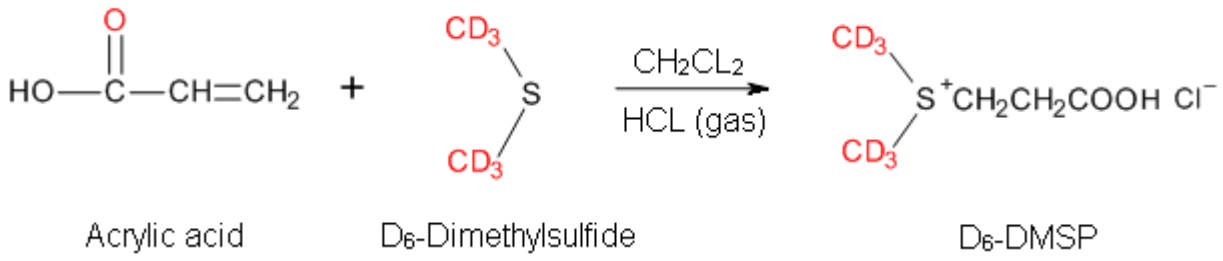


Figure 3.3. Synthesis of D_6-DMSP.

D_4-Homarine was made by dissolving 0.25 grams of D_4-picolinic acid (CDN Isotopes, Quebec, Canada) in 5 mL of MeOH (Figure 3.4). One gram of silver oxide (Ag_2O) was added and shaken until a grey solid appeared. One mL of iodomethane (CH_3I) was added, the mixture shaken and then refluxed at 65–70 ºC for three hours. The mixture was cooled and filtered three times with MeOH. The collected liquid was rinsed with 0.5 mL concentrated hydrochloric acid to remove any silver ions. The mixture was left to sit for twenty minutes and then re-filtered. The filtrate was dried down with isopropanol. Recovery of D_4-homarine (yield = 0.029 grams, 5.5%) was sufficient to be used as an internal standard for the assay. HPLC was used to verify that the product was D_4-homarine following the method of Storer et al. (2006) (Figure 3.5).

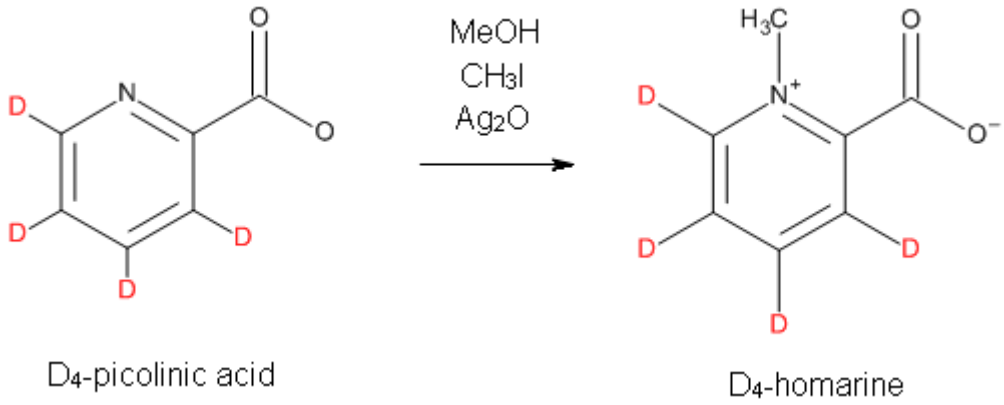


Figure 3.4. Synthesis of D_4-homarine.

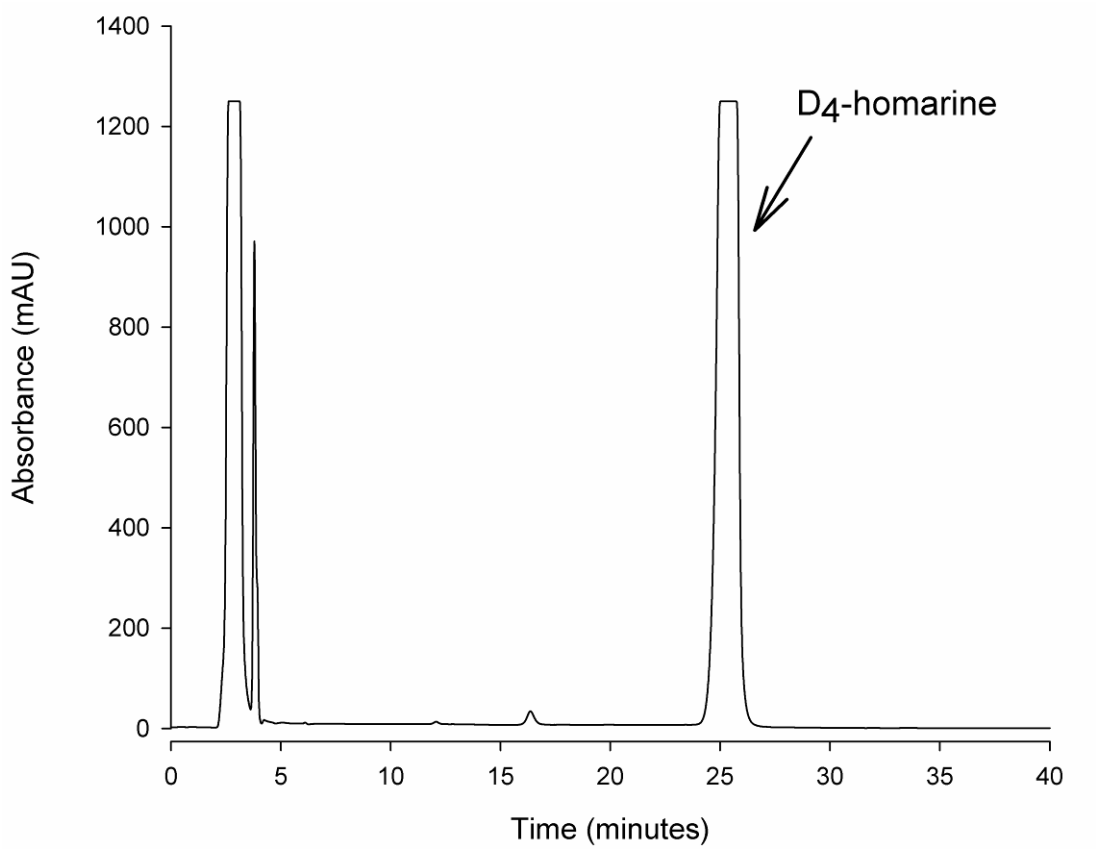


Figure 3.5. HPLC chromatogram of D_4-homarine at a retention time of 25.2 minutes.

3.3 Prey – method validation

3.3.1 Preliminary experiments

Initial efforts to analyse biomarkers using a modified extraction procedure described by de Zwart et al. (2003) and an LC-MS/MS assay developed for measuring biomarkers in serum (Lenky et al. 2012) provided unsatisfactory results. The extraction procedure appeared suitable for measuring biomarkers in seafood purchased locally from fish markets, but when it was applied to Antarctic prey samples there was a poor correlation between estimates of biomarkers measured by LC-MS/MS and ^{1}H NMR, especially for TMAO. The LC-MS/MS method recovered 20-50% less TMAO than the ^{1}H NMR assay described in Chapter Two on the same set of samples. There was also poor correlation with TMAO results that were obtained on the same set of Antarctic prey samples using a headspace-gas chromatography (HS-GC) assay developed at the Smithsonian National Zoological Park in Washington, DC (Eisert and Oftedal unpublished data). It appeared as though some Antarctic prey contained

endogenous compounds that interfered with the ionisation of TMAO, which was not observed in freshwater fish (salmon) or species that contained low concentrations of TMAO (squid and mussel). Since there was a good correlation between the TMAO results obtained by ^{1}H NMR and HS-GC (Passing-Bablock agreement test, $r = 0.993$, Figure 3.6), this suggested that there was an underlying problem with the LC-MS/MS method.

Unfortunately, the LC-MS/MS instrument used for this study is part of a large clinical laboratory where 1) an assortment of HPLC columns and buffers are used; 2) several types of analytes are screened for with a high sample throughput; and 3) different matrices including blood and raw urine are injected into the instrument, resulting in a high risk of contamination and deterioration of instrument performance as a result. After all research samples were already analysed, significant drift in signal response and intensity was discovered with the instrument. This was likely due to ionisation temperature changes or contamination of the ion source components of the mass spectrometer (*e.g.*, from injecting raw urine that contains compounds such as phosphates that may precipitate inside the instrument).

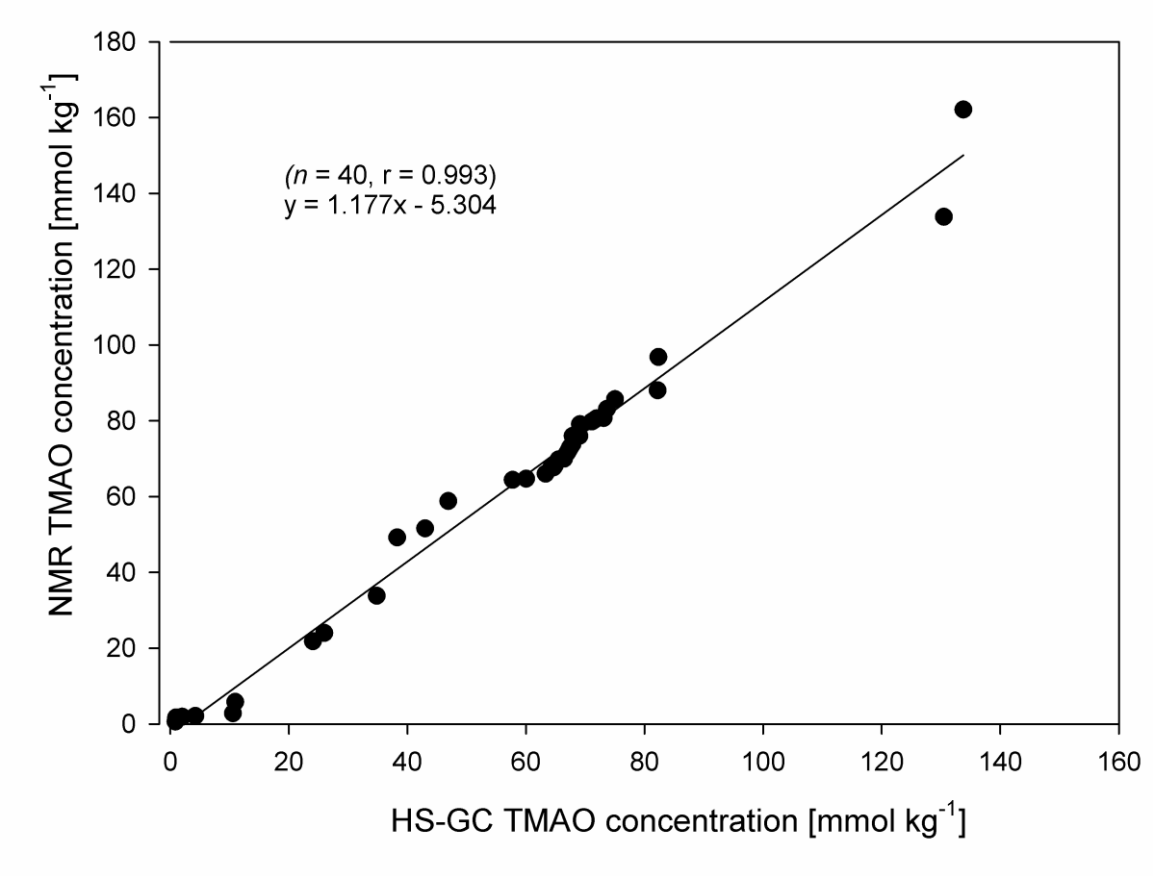


Figure 3.6. Method comparison of TMAO content in Antarctic prey.

Since the ^{1}H NMR procedure in Chapter Two gave results for TMAO that were consistent with HS-GC and GB was easily detected by ^{1}H NMR, a decision was made to only use LC-MS/MS for measuring AsB, DMSP and homarine in prey samples, which could not be easily detected by ^{1}H NMR.

3.3.2 Sample preparation

The PCA extracts from the Antarctic prey samples that were used to validate the ^{1}H NMR assay in Chapter Two were analysed on the LC-MS/MS. To determine whether perchlorate ions in the PCA extracts had any effect on the chromatographic profile, a 1 mL subsample of PCA prey extract was neutralised to a pH of ~ 7.0-7.4 with K_2CO_3 (measured by dropping a few µl of supernatant onto pH paper) and then centrifuged to remove the perchlorate ions. Additionally, Amberlite basic anion exchanger beads or Biorad AG 11 A8 resin were added to a 1 mL subsample of PCA prey extract until neutralised to a pH of ~ 7.0-7.4. This proved

to be a simpler and more time efficient method than adding K_2CO_3. Sharper peaks were observed with neutralised samples, but no difference was observed in the measured concentrations of AsB, DMSP and homarine between PCA and neutralised samples. Untreated PCA extracts were used for all subsequent analyses.

A 25 µL subsample of PCA extract from the various Antarctic samples that were prepared in Chapter Two, section 2.2.2 were combined with 500 µL of dilution solvent consisting of 90% ACN and 10% MeOH and 2 µM each of the internal standards $^{13}C_2$-arsenobetaine, D_6-DMSP and D_4-homarine. Samples were vortexed for 5 minutes and centrifuged at 13,000 rpm for 5 minutes. Then 200 µL was transferred to 96 well polypropylene plates for analysis on the LC-MS/MS. Plates were covered tightly with aluminium foil and kept at -20 °C until analysis to prevent sample evaporation or deterioration and run within 12 hours. Betaines have been shown to be stable in plasma and serum at 0 and 25 °C for at least 72 hours (Holm et al. 2003). Sampling for analysis was done on covered plates, as the autosampler was able to penetrate the foil cover.

3.3.3 LC-MS/MS instrumentation

Separation was performed on a Shimadzu Prominence (Kyoto, Japan) HPLC system. The flow rate was 400 µL min^{-1} using a gradient of two mobile phases. Solvent A contained 10 mM ammonium formate, 10 mM formic acid, 50% water and 50% ACN. Solvent B contained 90% ACN and 10% water. The gradient used for the analysis was: 0 minutes (initial) 50% A and 50% B, 6.50 minutes 100% A and 0% B, 6.60 minutes (final) 50% A and 50% B. All gradient steps were linear. Nitrogen was used as the collision gas. There was an interval of eight minutes between injections. Autosampler temperature was set to 10 °C to preserve sample quality during long runs and the injection volume was 10 µL. A Cogent 100 mm × 2.1 mm, 4 µm Diamond Hydride silica column (MicroSolv Technology Corporation, Eatontown, NJ, USA) was used, and the column oven temperature was set to 40 ºC. Samples were measured using an MDS Sciex API 4000 (Applied Biosystems, Mulgrave, VIC, Australia) tandem mass spectrometer with a turbo ion spray (ESI) probe. Analytes were measured in positive ion mode using multiple reaction monitoring (MRM). Mass transitions used for MRM are shown in Figure 3.7. The cycle time was 450 ms, and the ion source temperature was 350 ºC.

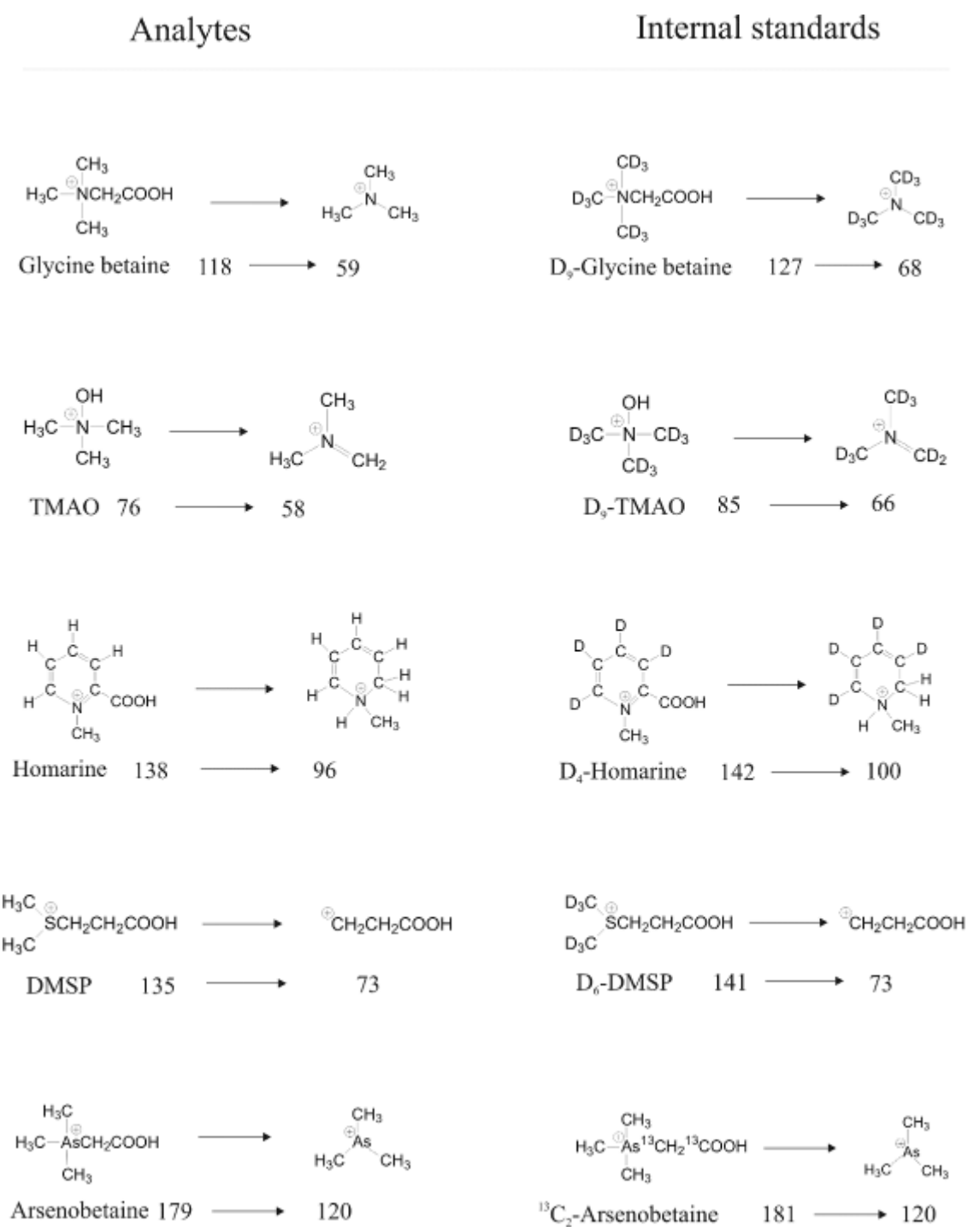


Figure 3.7. Mass transitions from the parent ion to the fragment ion used for detection in multiple reaction monitoring chromatograms.

To identify compound-specific mass spectrometer parameters, a compound optimisation was performed by infusing 2 µM standards of each analyte in 15 mM ammonium formate and ACN (75:25 by volume) into the mass spectrometer using the syringe pump at a flow rate of 10 µL min^{-1}. Compound specific parameters are given in Table 3.1. The HPLC system and mass spectrometer were controlled using Analyst software (Applied Biosystems).

Table 3.1. Compound-specific mass spectrometer parameters obtained for each analyte.

Analyte	Mass transition	DP	CE	CXP
GB	118 → 59	56	27	4
D_9-glycine betaine	127 → 68	61	27	4
TMAO	76 → 58	16	27	10
D_9-TMAO	85 → 66	91	29	2
Homarine	138 → 96	111	31	10
D_4-Homarine	142 → 100	81	31	6
DMSP	135 → 73	71	23	4
D_6-DMSP	141 → 73	56	23	4
AsB	179 → 120	111	29	8
$^{13}C_2$-Arsenobetaine	181 → 120	71	29	8

DP = decoupling potential, CE = collision energy and CXP = collision cell exit potential

The Cogent Diamond Hydride silica column separated the analytes sufficiently for detection by tandem mass spectrometry (Figure 3.8). Although DMSP and TMAO were coeluting, the monitoring of the different mass transitions ensured that they were not both present in the chromatograms.

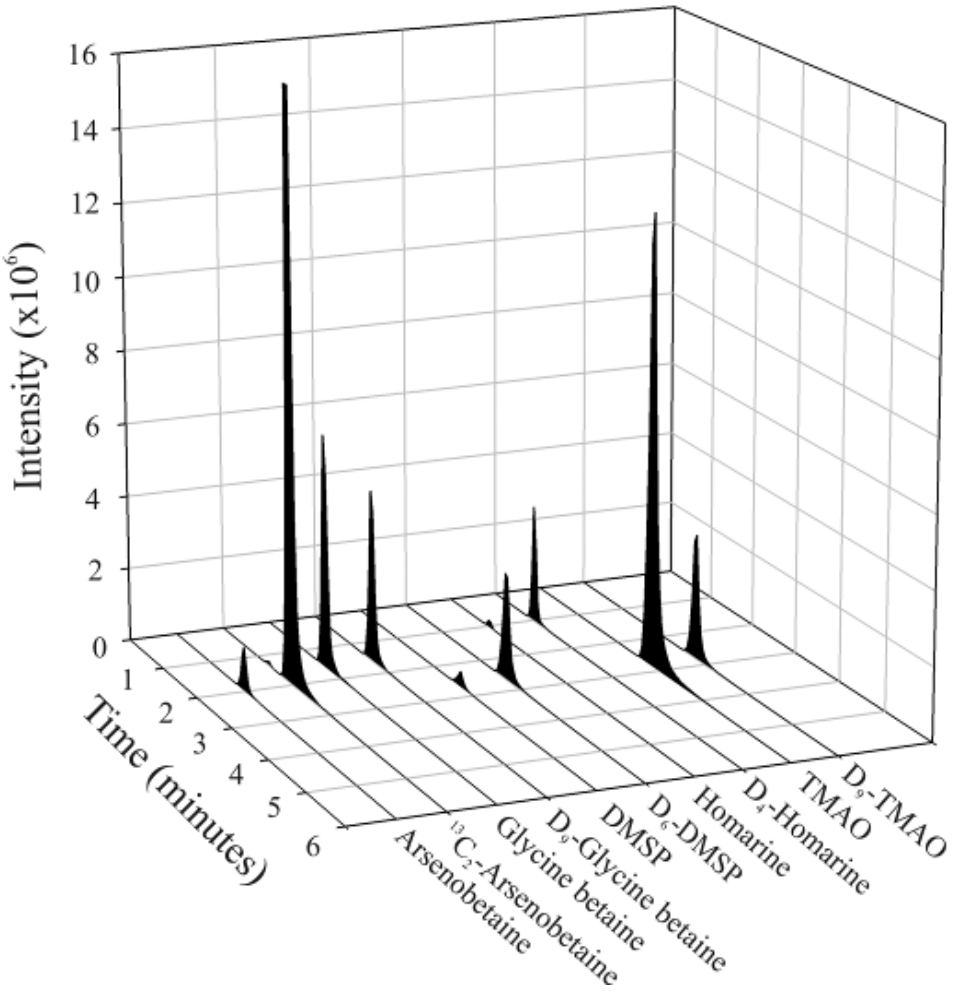


Figure 3.8. LC-MS/MS multiple reaction monitoring chromatograms of biomarkers and stable isotope-labelled internal standards in serum.

3.3.4 Calibration curve

To calibrate the prey data, aqueous standards of AsB, DMSP and homarine were prepared at concentrations of 1, 5, 10, 20, 40, 80 and 160 µmol L^{-1} (Figure 3.9). These standards were prepared by independent dilutions of a 10 mM aqueous stock standard for each analyte and multiple aliquots of each standard were stored at -80 °C in 1.5 mL microcentrifuge tubes until needed. Then 25 µL of standard was added to 500 µL dilution solvent and prepared as above to run on the LC-MS/MS. To quantitate each analyte, I calculated the peak area ratio (PAR) between the analyte (AsB, DMSP and homarine) divided by the corresponding stable isotope-labelled internal standard peak area ratio, and then plotted PAR against analyte concentration

to obtain a calibration curve. The linearity of the method was determined by weighted Deming linear regression.

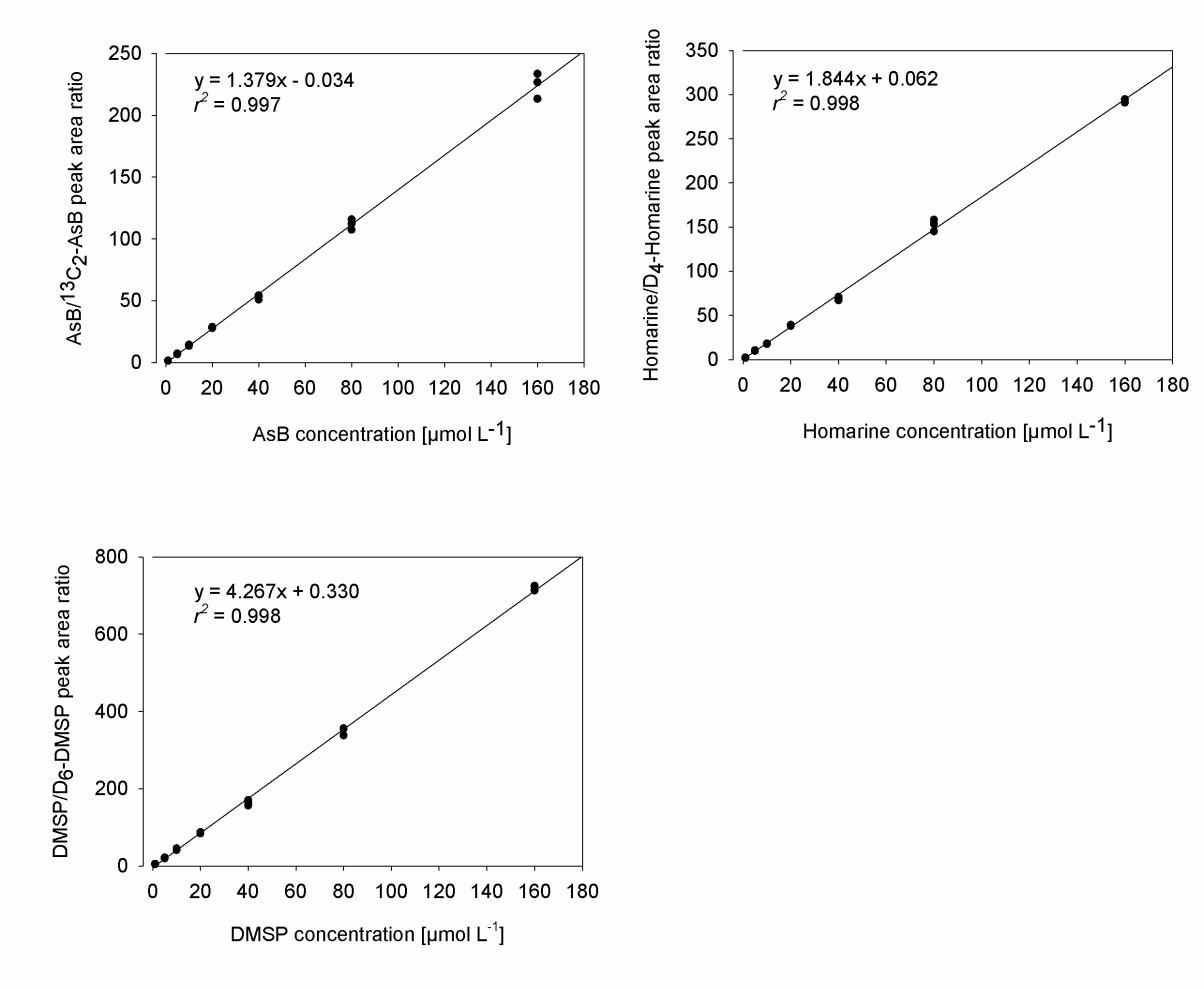


Figure 3.9. Standard curves used for calibrating prey data.

3.3.5 Instrument validation

Several sets of standards were analysed in the beginning, middle and end of each run to monitor and correct for instrument drift during each analytical run. QC samples (see below) were injected ten times at the beginning of each run in order to equilibrate the instrument and then every 15 samples to monitor drift. Low and high aqueous standards (20 and 80 µM) were also run every 15 samples to monitor drift and to allow correction of raw results for drift.

3.3.6 Precision and recovery

AsB, DMSP and homarine were added to salmon homogenate to obtain 'low' and 'high' QC material. These QC samples were used to determine recovery, analytical precision, and stability of the instrument. A piece of salmon fillet was chosen because it was not expected to contain DMSP and homarine, and only trace amounts of AsB (Amlund and Berntssen 2004). The salmon fillet was cut into small pieces and homogenised with a 1:1 ratio of distilled water using an Ultra Turrax to form a watery paste as decribed in Chapter Two, section 2.2.4. Low and high concentrations of 25 µmol L^{-1} and 100 µmol L^{-1} for AsB, DMSP and homarine from a 10 mM stock solution were then added to 1 gram subsamples of salmon homogenate, mixed, and extracted three times with 3 mL of 6% PCA and 1 mL DCM as described in section 2.2.4. Then 25 µL of extract was added to 500 µL dilution solvent and prepared as above to run on the LC-MS/MS. Six batches of five replicates (n = 30 samples) of salmon with both a low and high added level of the analytes were run by LC-MS/MS and the within-batch and between-batch CVs were calculated for each compound. For this assay to be acceptable, the precision at each QC level was required to be within 20% of the true value.

3.3.7 LOD and LOQ

LOD and LOQ were calculated using the same methods as in Chapter Two, section 2.2.5. Briefly, the LOD_b was calculated on aqueous standards using S/N = 3, while the LOD_s and LOQ_s for biomarkers in Antarctic prey were calculated using precision profiles. LOD_s was set at 20% RSD and LOQ_s at 15% RSD.

3.4 Results - prey

3.4.1 Signal drift correction

There was considerable drift in the signal response during the course of the analytical run for AsB, homarine and DMSP between the beginning and later calibration standards, as well as in salmon QCs and aqueous standards scattered throughout the run. The drift in signal response varied between the biomarkers and various statistical methods were employed to correct raw results for signal drift. An example of drift correction for homarine is discussed

below while statistical procedures for AsB and DMSP drift correction are provided in Appendix A.

A marked change in homarine signal response was observed between the first and later calibrations (Figure 3.10). There was little drift between the second and third calibrations, consistent with rapid drift during the first half of the run followed by relative stability in the signal response. The first calibration was linear but later calibrations became curvilinear (Figure 3.10).

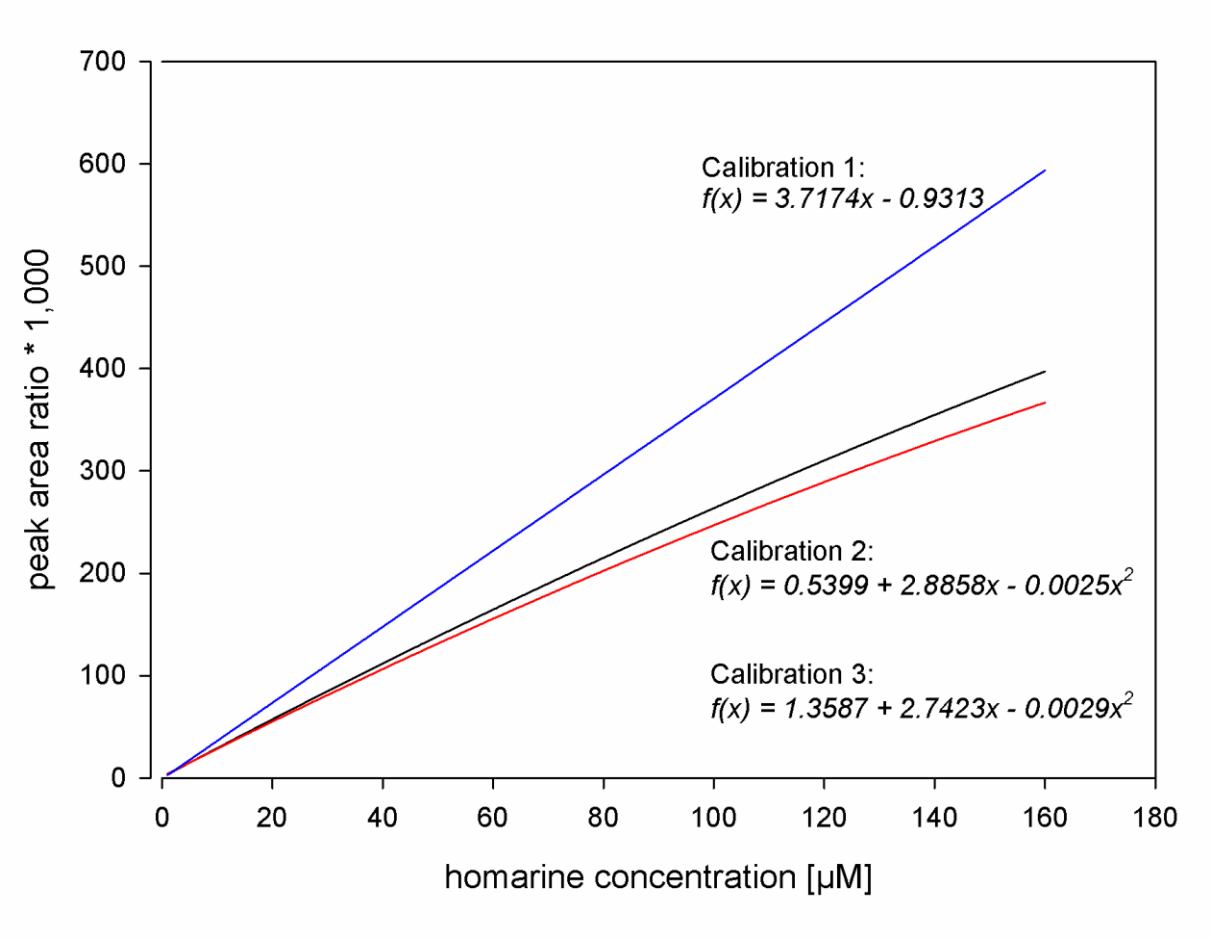


Figure 3.10. Change in signal response (external calibration function) between the initial (1) and later (2,3) calibrations.

It was hypothesised that drift (change in signal response to a sample of a given concentration, for example a standard or QC analysed in regular intervals) would be a function of time and follow an exponential decay curve (*Eq. 1*), and this model was confirmed by examining actual drift in standards and QCs. The theoretical initial signal at the beginning of the run (*i.e.*, in the absence of drift) can be estimated using *Eq. 1a*:

$$g(t) = y_0 + a \cdot e^{-kt} \qquad \textit{Eq. 1}$$

$$g(0) = y_0 + a \qquad \textit{Eq. 1a}$$

Because samples were injected into the instrument at regular intervals, vial sequence number (*t*) was used as a proxy for time (Figure 3.11). An exponential decay curve was modelled for each QC (25 and 80 µM) and standard (20 and 80 µM) separately to derive the initial signal value (*Eq. 1a*), and PAR were normalised by dividing them by this initial value, *g(0)*. It was apparent that the relative or fractional drift was similar in different QCs and standards and normalised data were therefore pooled to derive an estimate of overall drift for the run. An exponential decay function was then fitted to the pooled normalised data to derive an estimate of *k*, the decay constant for the relative change of signal with time (Figure 3.11).

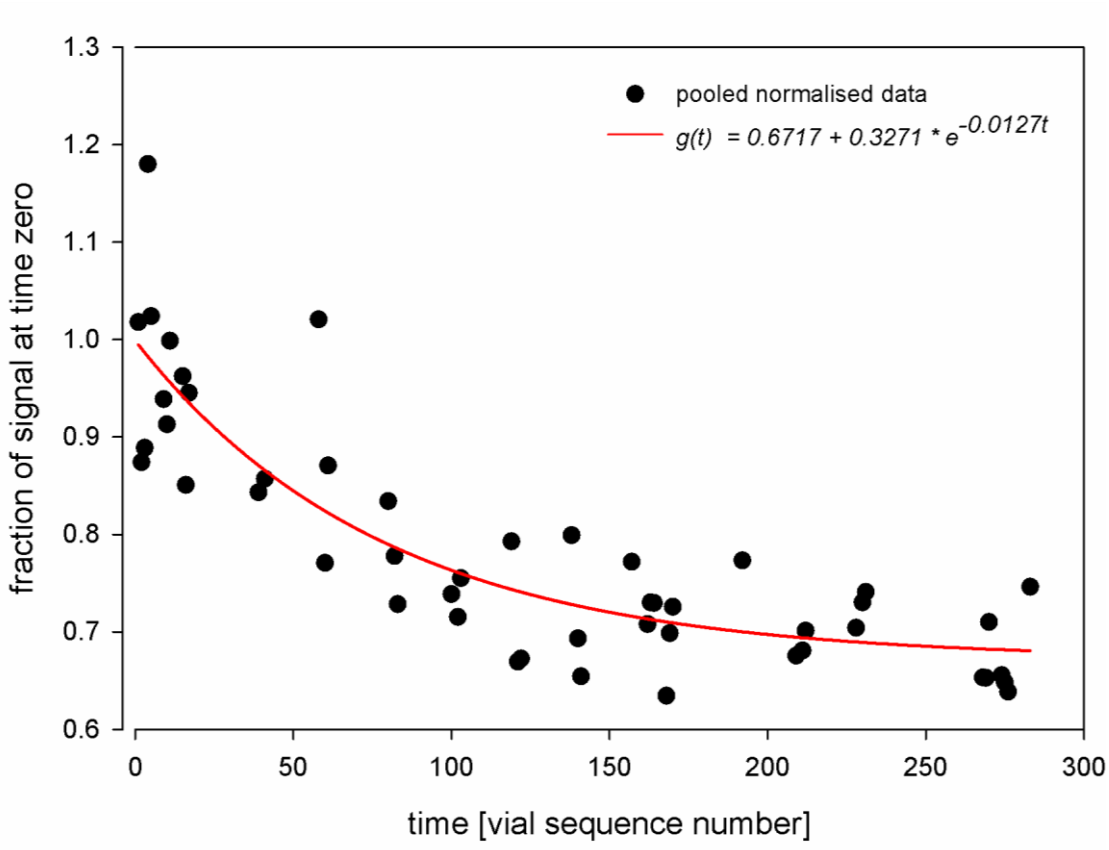


Figure 3.11. Pooled fractional drift in the normalised signal response of QC samples.

Separate exponential equations of the type shown in Figure 3.11, *g(t)* where *t* is the vial sequence number, were calculated for each analyte and used to calculate a drift correction factor. Measured PAR for sample replicates were divided by the drift correction factor to obtain the corrected PAR values.

Corrected PAR of homarine calibration standards (Figure 3.12) were used to calculate a pooled, corrected calibration function using weighted Deming linear regression analysis. This calibration function is shown in equation 2a where *t* is the vial sequence number and *x* is PAR:

$$f(x) = y0 + a(t) \qquad \text{Eq. 2}$$

$$f(x) = 7.839 + 3.822(t) \qquad \text{Eq. 2a}$$

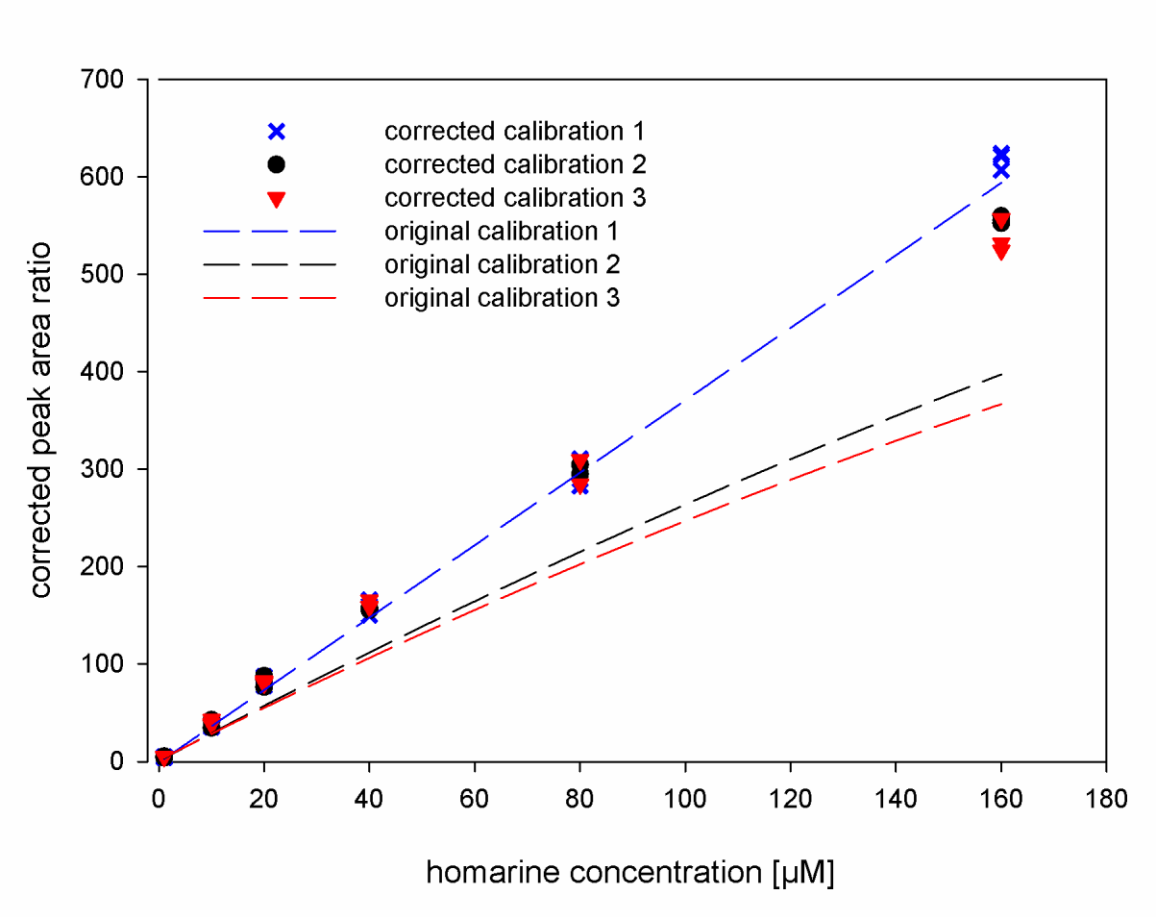


Figure 3.12. Drift-corrected calibration functions. The original calibration curves (dashed lines) are shown for comparison.

Parameters from this function were used to quantify all samples by applying slope and intercept values to the correct PAR results. As is apparent in Figures 3.12 and 3.13, the correction procedure resulted in a satisfactory correction of signal values, evident by good agreement between the first and later calibration curves after correction (Figure 3.12) and compensation of the progressive decline in signal value for QCs and standards (Figure 3.13).

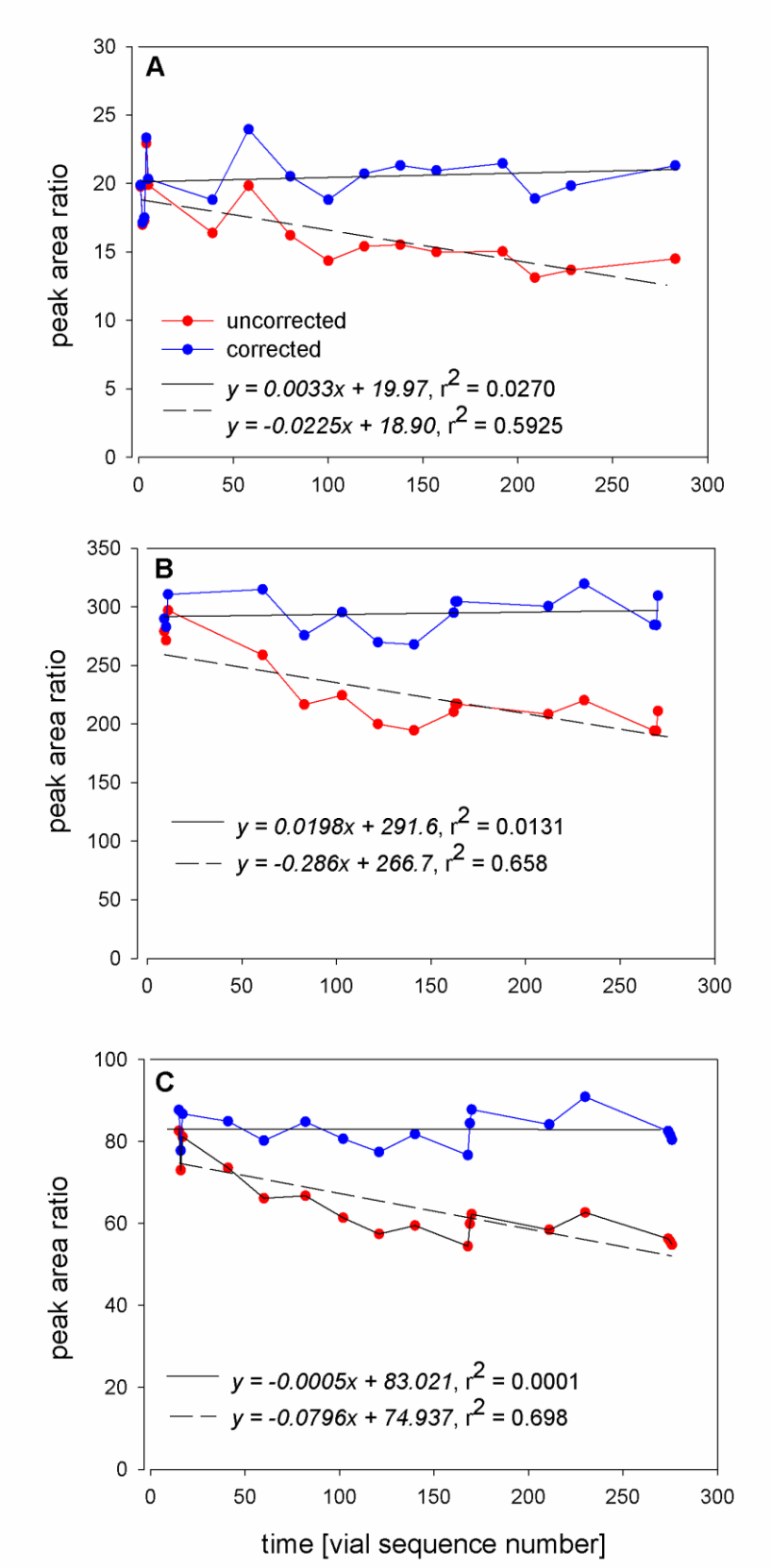


Figure 3.13. Uncorrected (red) and drift corrected (blue) peak area ratios for low QC, 25 µM (**A**) high standard, 80 µM (**B**) and low standard, 20 µM (**C**).

3.4.2 Precision and recovery

Precision and recovery were investigated at low and high concentrations of 25 µmol kg^{-1} and 100 µmol kg^{-1} for AsB, DMSP and homarine using salmon. The within-batch CV ranged from 3.5-7.3, and from 1.5-5.6 for the between-batch CV (Table 3.2). The analytical recovery was between 96 and 111%.

Table 3.2. Results of precision and recovery study of low and high added levels of biomarkers in salmon.

Analyte	Analyte added (µmol)	Mean[a] (µmol kg^{-1})	Within batch CV	Between batch CV	Recoveries (%)
AsB					
Low	25	20	4.8	5.6	111
High	100	103	3.5	5.0	
DMSP					
Low	25	27	7.3	4.8	110
High	100	109	4.5	1.5	
Homarine					
Low	25	25	4.7	1.7	96
High	100	97	4.3	3.0	

3.4.3 LOD and LOQ

The method detection limit using the S/N approach (LOD_b) and the LOD_s and LOQ_s derived from replicate precision profiles from Antarctic prey are provided in Table 3.3. Precision profiles were created by fitting a rational curve to the data using SigmaPlot (*e.g.*, Figure 3.14). The LOD_s was greater than the LOD_b for AsB, homarine and DMSP in Antarctic species, owing to a different sample matrix (Table 3.3).

Table 3.3. Limits of detection (LOD) and quantitation (LOQ) of biomarkers measured in aqueous standards (LOD_b, µmol L^{-1}) and in Antarctic prey matrix (LOD_s, LOQ_s, µmol kg^{-1}) by LC-MS/MS.

Analyte	LOD_b (S/N = 3 in aqueous sample)	LOD_s (20% RSD in prey)	LOQ_s (15% RSD in prey)
AsB	0.06	3.0	3.4
Homarine	0.9	3.2	5.0
DMSP	0.3	3.6	6.8

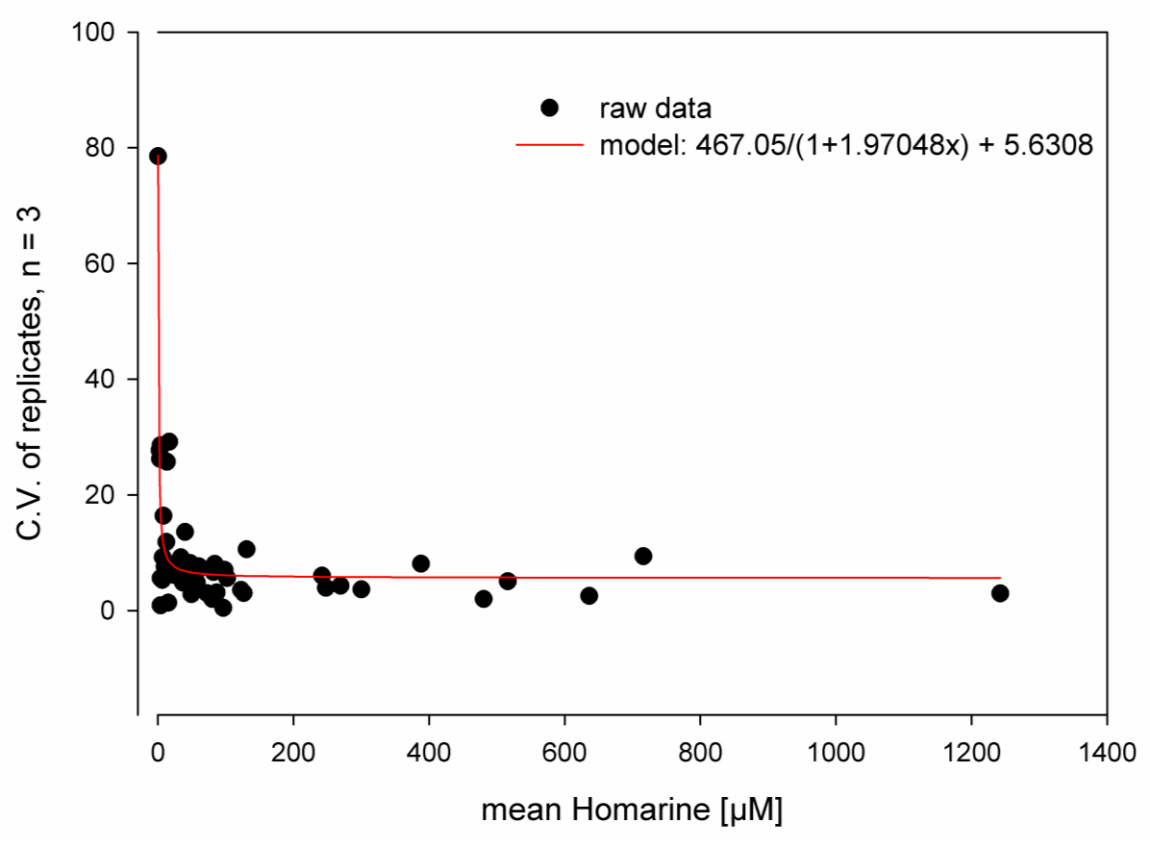


Figure 3.14. Precision *versus* concentration for homarine in Antarctic prey.

3.4.4 Method comparison

The results obtained for AsB were compared to those obtained previously using GF-AAS on the same set of Antarctic samples (Eisert and Oftedal, unpublished data). Even though drift corrections had to be applied to the LC-MS/MS data due to instrument issues, there was good agreement between the two methods. GF-AAS detected about 20% more AsB in samples than LC-MS/MS (Passing Bablock agreement test, r = 0.969, Figure 3.15).

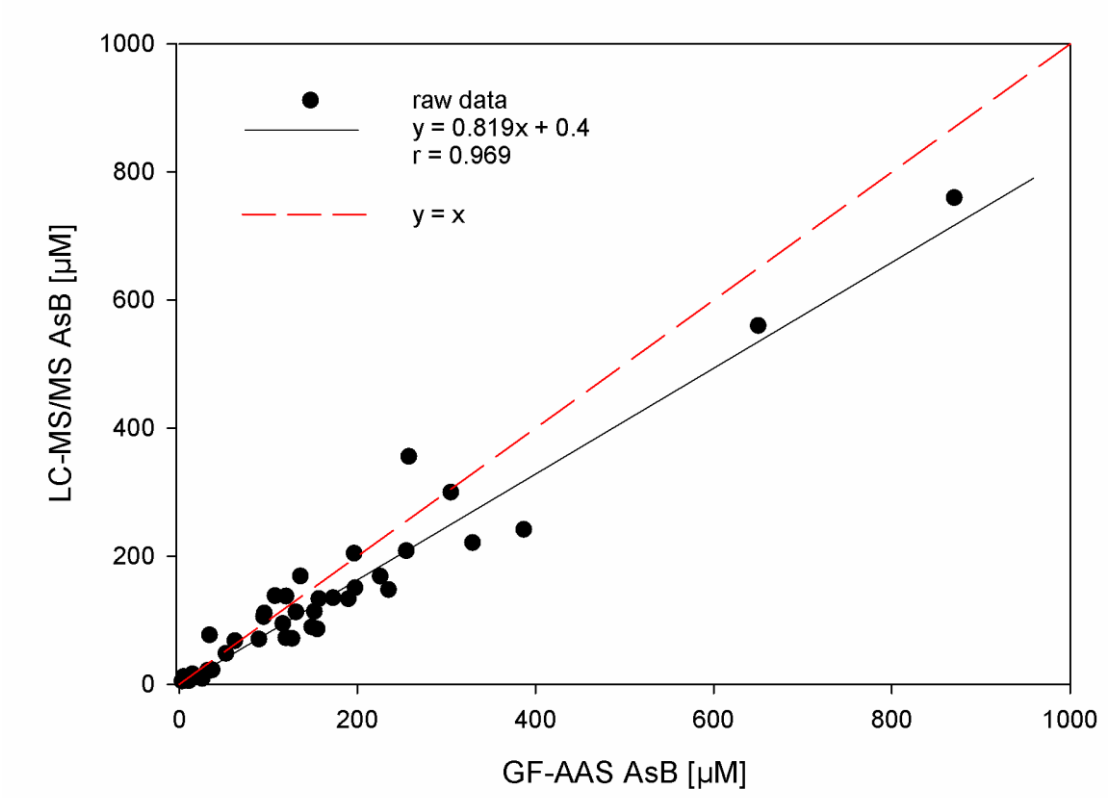


Figure 3.15. Method comparison of AsB concentration in Antarctic prey.

3.5 Bovine serum – method validation for measurement of biomarkers in blood plasma

3.5.1 Sample preparation

Method validation for detecting and quantifying all biomarkers in seal plasma was carried out using bovine serum because it was readily available as a standardised reagent and because it was expected to a) be sufficiently similar to seal plasma and b) not contain any of the

biomarkers of interest, except GB. Bovine serum (25 µL) was added to 500 µL of dilution solvent which consisted of 90% ACN and 10% MeOH containing 2 µM of each of the internal standards (D_9-glycine betaine, D_9-TMAO, D_4-homarine, D_6-DMSP and $^{13}C_2$-arsenobetaine). Samples were vortexed for 5 minutes and centrifuged at 13000*g* for 5 minutes, then 200 µL was transferred to 96 well polypropylene microtitre plates for analysis as described in section 3.3.2. Concentrations of each analyte in bovine serum were determined by calculation of the ratio of each analyte's peak area to their corresponding stable isotope-labelled internal standard peak area as described in section 3.3.4. Unknown samples were quantitated using external calibration curves as described above and in the next section.

3.5.2 Calibration curve

To calibrate the data, aqueous standards were prepared to reflect expected concentrations in seal plasma: for TMAO and GB, concentrations at 1, 5, 10, 20, 40, and 80 µmol L^{-1}, AsB at concentrations of 0.05, 0.1, 0.25, 0.5, 1, 2 and 5 µmol L^{-1}, DMSP at concentrations of 0.25, 0.5, 1, 2 and 5 µmol L^{-1} and homarine at concentrations of 0.1, 0.25, 0.5, 1, 2, 5 and 10 µmol L^{-1}. These standards were prepared by independent dilutions of a 10 mM aqueous stock standard for each analyte, split into multiple aliquots, and stored at -80 °C in 1.5 mL microcentrifuge tubes until needed. Then 25 µL of standard was added to 500 µL dilution solvent and prepared as above (section 3.5.1) to run on the LC-MS/MS. The TMAO, GB, AsB, DMSP and homarine peak area was divided by the peak area of the corresponding stable isotope-labelled internal standard peak area and plotted against analyte concentration to obtain calibration curves (Figure 3.16).

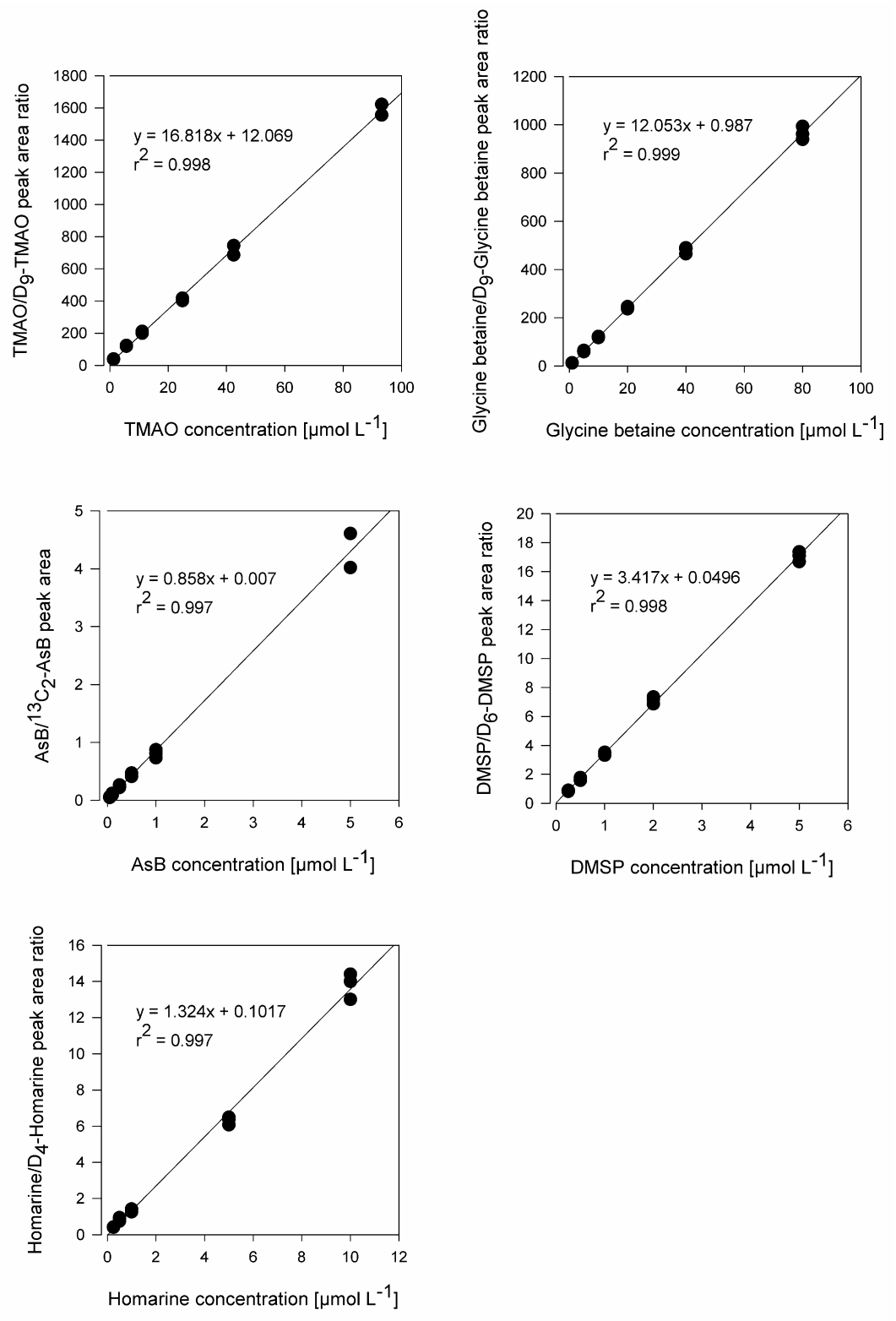


Figure 3.16. Standard curves used for calibrating bovine serum data.

3.5.3 Dilution experiment

In the study by Eisert et al. (2005), female Weddell seals were found to contain plasma TMAO levels greater than 600 µmol L^{-1}, which is outside the linear range of the TMAO calibration curve for LC-MS/MS (Figure 3.17 and additional data not shown). Validation experiments were carried out in order to dilute serum TMAO concentrations into the linear range of the assay and determine whether there was a dilution effect on the samples. Bovine serum (10 mL) was spiked with TMAO to a concentration of 70.5 µmol L^{-1} TMAO. This sample was diluted gravimetrically with 100 mM saline at dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:100. Samples were prepared as described in section 3.5.1 and run on the LC-MS/MS. The results of the dilution experiment are provided in Figure 3.17.

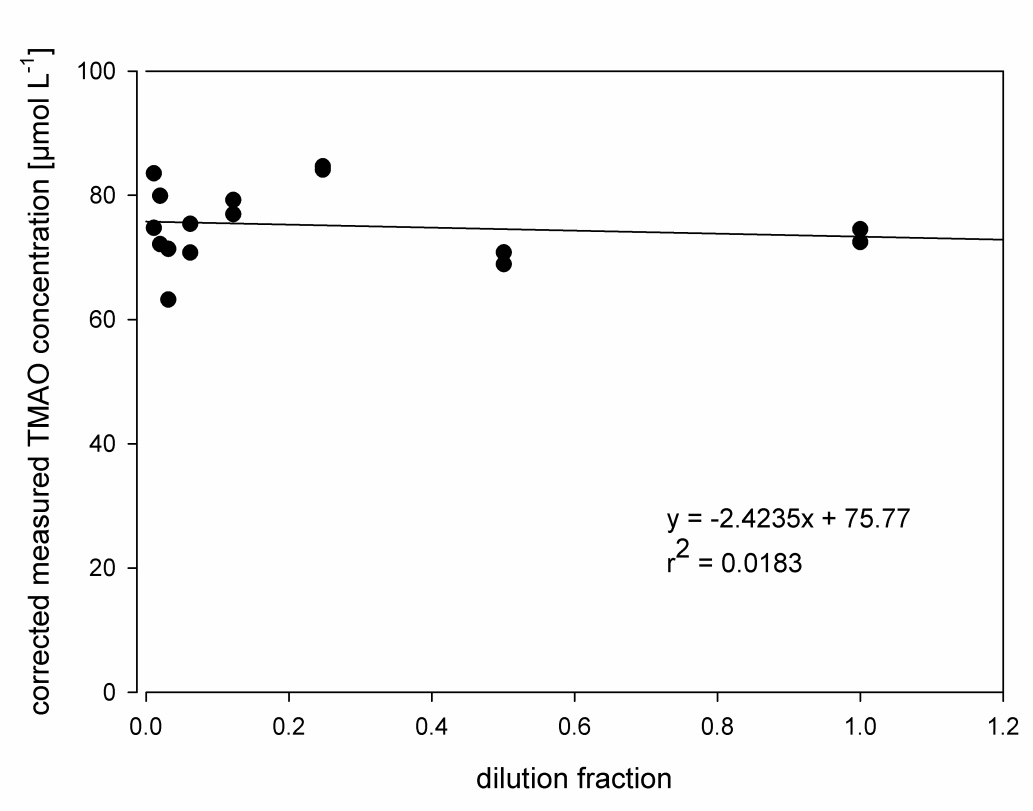


Figure 3.17. Plot of dilution *versus* corrected concentration (measured concentration/dilution) for TMAO in bovine serum.

3.5.4 Instrument validation

Standards and QCs were used to equilibrate the instrument and monitor drift as described in section 3.3.5.

3.5.5 Precision and recovery

TMAO, GB, AsB, DMSP and homarine were added to bovine serum to obtain 'low' and 'high' QC materials. These QC samples were used to determine recovery and analytical precision. Six batches of five replicates (n = 30 samples) of bovine serum with both a low added level of the analytes and a high added level of the analytes were run by LC-MS/MS and the within batch and between batch CVs were calculated for each compound. For the assay to be acceptable, the precision at each QC level was required to be within 20%.

3.5.5 LOD and LOQ

LOD and LOQ were calculated using the same methods as in section 3.3.7 except precision profiles were created from biomarker data obtained from Weddell seal plasma data in Chapter Five.

3.6 Results – bovine serum

3.6.1 Drift correction

Drift corrections were only applied to AsB and TMAO because there was no observed drift for the other biomarkers. Statistical procedures are provided in Appendix A.

3.6.2 Precision and recovery

The results of the precision and recovery study for each biomarker in serum are shown in Table 3.4. Precision and recovery were investigated at low and high concentrations of 50 µmol L^{-1} and 200 µmol L^{-1} for TMAO and GB and 10 µmol L^{-1} and 30 µmol L^{-1} for AsB,

DMSP and homarine in serum. The within-batch CV ranged from 3.5-8.1 and from 0.9-6.0 for the between-batch CV (Table 3.4). Mammals have endogenous GB in plasma, which explains the high level of GB present in the low spiked sample (Table 3.4). Low levels of TMAO in bovine serum are likely to orginate from bacterial degradation of betaines and other similar compounds in the rumen. The analytical recovery was between 85 and 95%.

Table 3.4. Results of precision and recovery study of low and high added levels of biomarkers in bovine serum.

Analyte	Analyte added	Mean[a] (µmol L^{-1})	Within batch CV	Between batch CV	Recoveries (%)
GB					
Low	50	245	3.6	1.9	
High	200	372	4.0	2.8	85
TMAO					
Low	50	70	4.4	2.4	
High	200	200	4.6	0.9	87
AsB					
Low	10	9	3.5	6.0	
High	30	28	3.6	2.9	93
DMSP					
Low	10	9	4.0	2.7	
High	30	28	4.6	1.8	95
Homarine					
Low	10	9	8.1	3.7	
High	30	26	7.7	4.5	86

[a] Mean (of triplicates) baseline concentrations in bovine serum without added levels of the analytes were 187 µmol L^{-1} GB and 14.2 µmol L^{-1} TMAO. AsB, DMSP and homarine were not detected.

3.6.3 LOD and LOQ

The estimated LOD_s and LOQ_s using precision profiles and the instrument LOD_b are provided in Table 3.5. The LOD_s and LOQ_s could not be calculated for DMSP or GB because DMSP was not detected in any Weddell seal plasma samples, and GB was present in relatively high concentrations in all samples analysed (range 8 to 40 µmol L^{-1}, Figure 3.18).

DMSP was reliably detected in aqueous standards at 0.1 µmol L^{-1} and in plasma QC's at 0.5 µmol L^{-1} so the LOD for DMSP using this assay was estimated to be between 0.1 – 0.5 µmol L^{-1}. Holm et al. (2003) calculated the LOD for GB in human plasma as 0.3 µmol L^{-1}. LOD_s and LOD_b for AsB in plasma were the same, while the LOD_s increased for TMAO and homarine in plasma, presumably due to matrix effects (Table 3.5). Homarine was also reliably detected in aqueous standards at 0.1 µmol L^{-1} and in plasma QC's at 0.5 µmol L^{-1}, which is below the calculated LOD_b.

Table 3.5. Limits of detection (LOD) and quantitation (LOQ) in µmol L^{-1} of biomarkers determined in aqueous standards and in Weddell seal plasma matrix using LC-MS/MS. NC = not calculable.

Analyte	LOD_b (S/N = 3 in aqueous sample)	LOD_s (20% RSD in plasma)	LOQ_s (15% RSD in plasma)
TMAO	0.04	0.51	0.62
AsB	0.06	0.06	0.10
Homarine	0.9	1.4	1.9
DMSP	0.3	N/C	N/C
GB	0.04	N/C	N/C

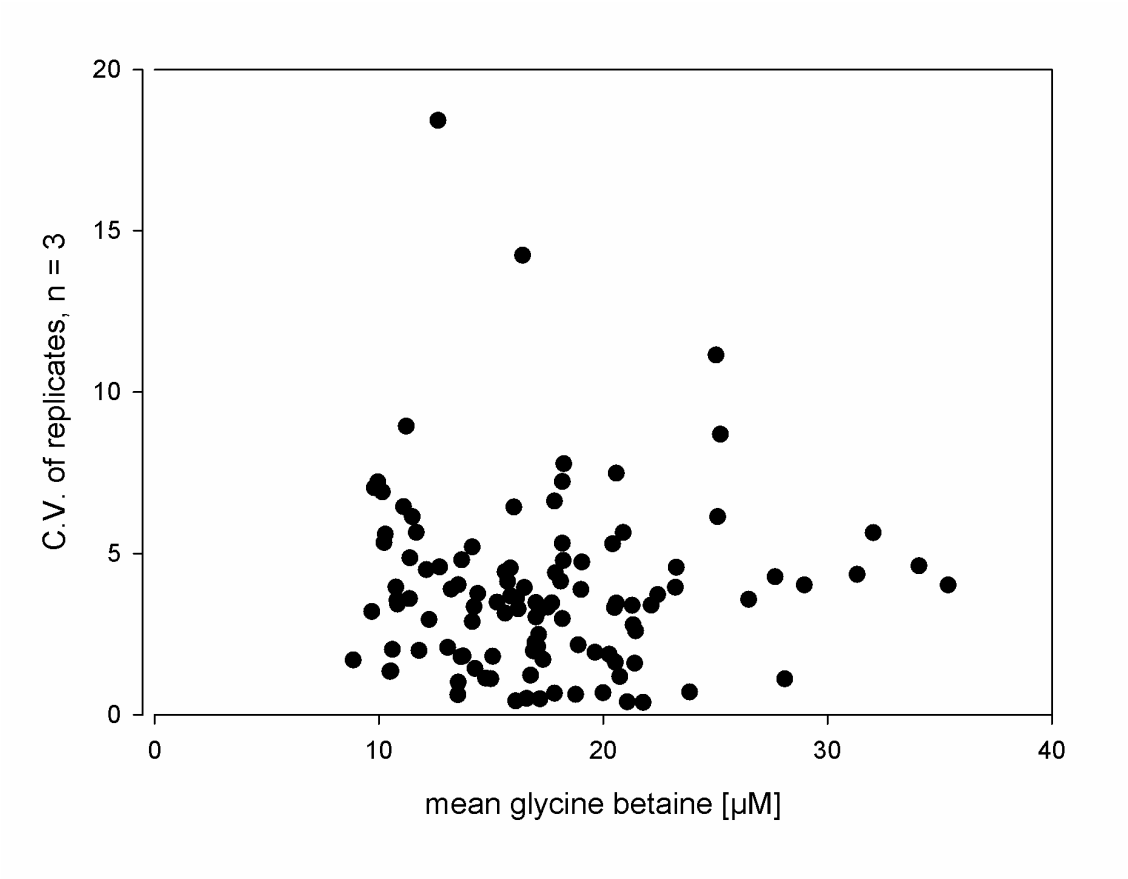


Figure 3.18. Precision *versus* concentration for glycine betaine in Weddell seal plasma. Note there is no consistent decline in replicate C.V. with concentration (see Figure 3.14) due to the limited range of concentrations found in Weddell seal plasma samples.

3.7 Discussion

This is the first study to measure homarine and AsB using LC-MS/MS and the first to use D_4-homarine and $^{13}C_2$-arsenobetaine as internal standards. Sample preparation is simple, and one of the major advantages of measuring samples by LC-MS/MS over HPLC or GC is that these biomarkers can be simultaneously detected and measured with a throughput of ~200 samples over a twenty-four hour time period without extensive sample preparation or derivisation. Previously, Holm et al. (2003) measured choline, betaine and DMG by MS/MS after separation on a normal-phase silica column. Sharper peaks were observed when using the Cogent Diamond Hydride column rather than an unmodified silica column of similar dimensions (data not shown).

The LC-MS/MS procedure that was initially developed was suitable for analysing biomarkers in locally purchased seafood and bovine serum, but not for measuring biomarkers in Antarctic prey samples. Low recoveries were observed for all analytes, but especially for TMAO. The sensitivity and accuracy of an analytical procedure can be affected by co-eluting compounds in the sample matrix. It was initially thought that urea was an interfering matrix component, but Antarctic fish do not contain high amounts of urea (Raymond and DeVries 1998). The assay was then optimised for detecting AsB, DMSP and homarine in prey by making minor adjustments to the procedure and applying more selective sample preparation techniques. These included injection of smaller sample volume from 10 to 5 µl, a more dilute sample (increasing the extraction solvent: sample ratio from 1:10 to 1:20), decreasing the amount of stable isotope-labelled internal standard from 10 µM to 2 µM and increasing the scan time from 4.5 to 6.5 minutes. The risk of interference from matrix components increases with decreasing scan time because the chromatographic separation ability of the instrument decreases (Jemal 2000). Therefore, it is important to find a balance between a run time that allows sufficient separation of the analytes without compromising the overall sample throughput. For this research, a longer scan time was favoured over sample throughput due to the problems encountered with the instability of the instrument.

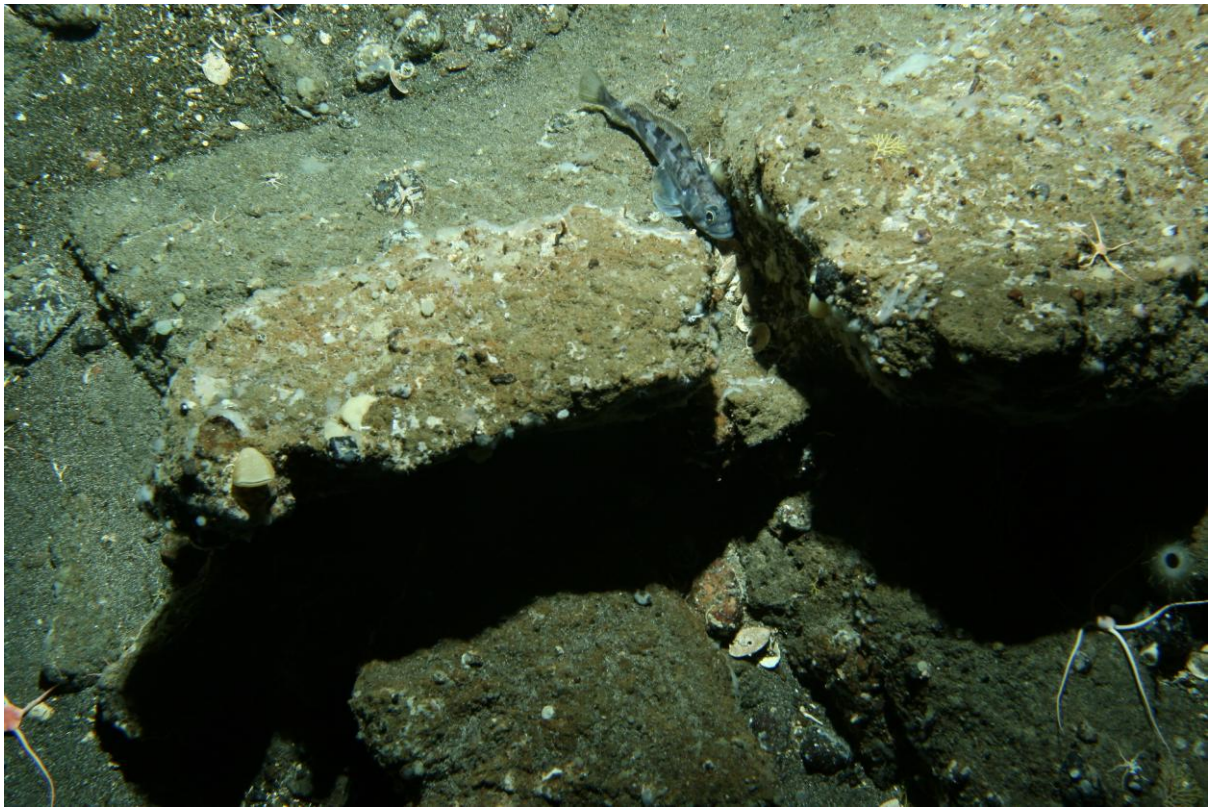
LC-MS/MS methods that use ESI (as in the case for this research) are likely to experience matrix effects (Annesley 2003, 2007). The presence of less volatile compounds can change the efficiency of droplet formation or evaporation which in turn affects the amount of charged ions in the gas phase that reaches the detector (Annesley 2003). Therefore, in order to achieve good recoveries, it is important to have isotopically labelled internal standards for each analyte (Holm et al. 2003, Kirsch et al. 2010). A stable isotope-labelled internal standard will not eliminate a matrix effect, but it will greatly reduce interference by matrix components. However, in this study there was a gradual change in the response of both the internal standard and analyte over time. In some cases, peak areas of internal standards and analytes changed in opposite directions, changing the actual analytical signal that consists of the peak area ratio of analyte: internal standard. To overcome this issue, (1) QCs were run prior to the start of the main run in order to ensure that the instrument had stabilised prior to analysing the main set of prey or plasma samples, and (2) QCs and standards were scattered throughout the run to monitor the instruments performance and provide sufficient data to allow drift correction. Even though a drift problem had not been reported previously with any other

current users of the LC-MS/MS instrument used in this study, this is possibly due to the fact that other users do not routinely include repeated calibrations and QCs to monitor potential drift during analytical runs. Drift and calibration instability appears to be a problem encountered elsewhere with LC-MS/MS (Sangster et al. 2006, Gika et al. 2007).

The assay developed here for the analysis of Weddell seal feeding activities also has potential uses for measuring biomarkers in blood, tissues, urine, and the human diet for physiological, nutritional and clinical studies. GB is an important osmolyte in most animals and many plants, and also has an important role in homocysteine metabolism in mammals (Lever and Slow 2010, Ueland 2011). There is also an increasing interest in the importance of dietary glycine betaine and its effect on performance enhancement in athletes (Hoffman et al. 2009). TMAO has been recently implicated in vascular disease (Wang et al. 2011) and is measured along with trimethylamine for diagnosing fish odour syndrome (Mackay et al. 2011). AsB is excreted rapidly in human urine after seafood consumption and is generally thought to be non-toxic (Edmonds and Francesconi 1993), and measuring it is a definitive method to distinguish between non-toxic cationic arsenic and toxic anionic forms of arsenic. DMSP is important in environmental chemistry as the source of DMS, the "smell of the sea", that is also formed rapidly after animal death leading to an undesirable product through foul odour/flavour production (Smit et al. 2007). While most studies measure DMSP by converting it first to DMS (Hill et al. 1995, Tang et al. 1999), the method presented here allows for direct quantification of DMSP in biological samples (Li et al. 2010). The assay described in this Chapter can also be applied to measure other analytes such as proline betaine, trigonelline and choline in foods (de Zwart et al. 2003). Thus this assay will have a wide range of potential applications in addition to the principal aim of determining the feeding behaviour of Weddell seals (Chapter Five).

Chapter Four

Distribution of TMAO, AsB, GB, homarine and DMSP in Antarctic marine species and their applications as dietary biomarkers



Seabed image of the Ross Sea. Photo provided by M. Pinkerton, NIWA.

4.1 Introduction

Many aquatic organisms accumulate low molecular weight organic compounds such as methylamines, amino acids, urea, polyols and sugars in their cells and tissues when they are under osmotic stress from high salt concentrations, freezing, high temperatures, increased pressure or dehydration (Yancey 2005). With the exception of urea, these compounds are often termed "compatible" osmolytes because they are accumulated at relatively high concentrations without having a perturbing effect on macromolecules such as proteins and nucleic acids (Yancey et al. 1982).

Although osmolytes are common to all living organisms, there is now widespread evidence that considerable variation exists in the osmolyte systems used across taxa (Yancey 1994, 2005). For example, Carr et al. (1996) showed that the osmolytes used varies amongst fishes, crustaceans, and molluscs. Fish use predominately creatine, lactate and TMAO, molluscs use predominantly taurine, GB and TMAO, and crustaceans use mainly glycine, GB and TMAO. However, even within the same taxon, osmolyte composition differs. Among pelecypod molluscs, taurine and GB are dominant osmolytes while taurine, glycine and alanine are dominant in the Pacific razor clam *Siliqua patula*. Carr et al. (1996) found even greater variability among cephalopods. GB and taurine were the two dominant osmolytes in the octopus *Octopus dofleini* and in one species of squid *Illex argentinus* while GB and TMAO were dominant in the squid *I. illecebrosus* and proline betaine and glycine were dominant in the squid *Loligo vulgaris*. A number of studies by Professor Paul Yancey and his group at Whitman College (Washington, USA) have also shown that deep-sea animals have different osmolyte compositions than shallow-water species (Gillett et al. 1997, Kelly and Yancey 1999, Yancey et al. 2001). For example, shallow-water invertebrates are most often dominated by taurine and glycine. Deep sea echinoderms (sea cucumbers and sea urchins) have high levels of the polyol scyllo-inositol, while some deep sea gastropods and polychaetes have high levels of betaine and glycerophosphocholine (Yancey et al. 2002, Yancey 2005). Osmolytes in *Riftia* species (tubeworms) from hydrothermal vents at 2.6 km depth are dominated by hypotaurine (Yin et al. 2000).

Much of the research on osmolytes has been on their compatibility with cellular function and protective properties. A novel and uninvestigated approach is using osmolytes to determine trophic relationships. Marine fish and invertebrates contain several organic osmolytes that are

not utilised by mammals and therefore are potentially useful as dietary biomarkers. Eisert et al. (2005) found that diet-derived levels of TMAO and AsB, an arsenic analogue of GB, are present in lactating Weddell seal plasma after feeding. These compounds function as dietary biomarkers because they are only detected in the bloodstream if the seals are feeding. Presently, their detection in Weddell seal plasma can tell us whether a female is feeding or fasting, but our understanding of biomarker distribution within Weddell seal prey species and their trophic accumulation is limited.

Investigating osmolytes across trophic levels should be straightforward because the Antarctic food web is relatively simple (Hempel 1985). However, there is little published information on osmolyte distribution within the Antarctic ecosystem. Only a few studies have been conducted on a limited number of organisms (Oehlenschläger 1991, Raymond and Devries 1998, Grotti et al. 2010) and a comprehensive analysis of all osmolytes was not completed in these studies, especially of those osmolytes that are potential biomarkers. TMAO has been identified in the white muscle of four teleost fish from the sub-Antarctic (Oehlenschläger 1991) while Raymond and DeVries (1998) determined the TMAO content in tissues and serum of eight teleost fish from McMurdo Sound. Grotti et al. (2010) determined AsB in the extracts of nine marine Antarctic organisms, mainly invertebrates; however, this study focused on the different chemical forms of arsenic rather than on osmolyte function. Homarine has been isolated from the gastropod *Marseniopsis mollis,* which causes feeding deterrence in its main predator, the sea star *Odontaster validus*. Surprisingly, homarine was not detected in the main prey of *M. mollis*, the ascidian *Cnemidocarpa verrucosa* (McClintock et al. 1994), which suggests that *M. mollis* aquires homarine from another source, or synthesises homarine directly. The phytoplankton community of McMurdo Sound and the southern Ross Sea is dominated by the haptophyte *Phaeocystis antarctica* which is responsible for high levels of DMSP production (Ditullio and Smith 1996, Arrigo et al. 1998, Rellinger et al. 2009). Elliot et al. (2009) detected DMSP in mesozooplankton that graze on *Phaeocystis antarctica* in McMurdo Sound, while elsewhere *P. antarctica* has been found in stomachs of Antarctic krill and other crustaceans (Nejstgaard et al. 2007) which suggests these species will also contain DMSP. To date, DMSP has not been analysed in chordates or molluscs and no studies have examined GB distribution within the Antarctic ecosystem.

The aim of this study was to collect a variety of Antarctic marine species (Table 4.1) to determine how much overall variation was present for individual biomarkers. TMAO, GB,

AsB, homarine and DMSP were screened for in prey and potential prey of the Weddell seal in order to (1) define taxon-specific biomarker patterns, and (2) determine whether they could be used for differentiating amongst different prey types. To do this, ^{1}H NMR was employed to analyse TMAO and GB, while LC-MS/MS was utilised for determining AsB, homarine and DMSP (see Chapters Two and Three).

Table 4.1. Habitat and feeding characteristics of Antarctic marine organisms analysed in this study.

Family and Species	Habitat	Diet	Reference
Fish			
Nototheniidae			
Dissostichus mawsoni (Antarctic toothfish)	Pelagic	Fish, mysids, cephalopods and amphipods	Eastman (1985a), Eastman (1985b), Bury et al. (2008)
Lepidonotothen squamifrons (grey rockcod)	Benthic	Diet not known	
Pagothenia borchgrevinki (bald notothen)	Cryopelagic	Pteropods, copepods, hyperiids, euphausiids, mysids, larval *P. antarcticum*	Eastman (1985b), Foster and Montgomery (1993), Montgomery et al. (1993)
Pleuragramma antarcticum (Antarctic silverfish)	Pelagic	Zooplankton, copepods, euphausiids, mysids, amphipods, pteropods, polychaetes, larval *P. antarcticum*	Dewitt and Hopkins (1977), Eastman (1985b)
Trematomus bernacchii (emerald rockcod)	Benthic	Polychaetes, molluscs, isopod, mysids echinoderms, fishes, pteropod, copepods, euphausiids	Eastman (1985b), Foster and Montgomery (1993), Montgomery et al. (1993), Vacchi et al. (2000), La Mesa et al. (2004a)
Trematomus eulepidotus (blunt scalyhead)	Epibenthic	Euphausiids, *P. antarcticum*	Eastman and Hubold (1999)
Trematomus hansoni (striped rockcod)	Benthic	Polychaetes, molluscs, isopods, mysids echinoderms, fishes, pteropods, copepods, euphausiids	Eastman (1985b), Foster and Montgomery (1993), Montgomery et al. (1993)
Trematomus lepidorhinus (slender scalyhead)	Epibenthic	Amphipods	Takahashi and Nemoto (1984)
Trematomus pennellii (Sharp-spined notothen)	Benthic	Polychaetes, molluscs, isopods, mysids echinoderms, fishes, pteropods, copepods, euphausiids	Eastman (1985b), Foster and Montgomery (1993), Montgomery et al. (1993)
Trematomus scotti	Benthic	Diet not known	
Deap sea smelt - Bathylagidae			
Bathylagus antarcticus	Benthic	Polychaetes, chaetognaths, copepods, ostracods, amphipods, pteropods, salps; not found in McMurdo Sound	Hopkins and Torres (1989), Donnelly et al. (1990), Lancraft et al. (1991), Geiger et al. (2000)
Lanternfish - Myctophidae			
Electrona antarctica	Oceanic, Mesopelagic	Euphausiids, copepods, amphipods, salps, ostracods, pteropods; not found in McMurdo Sound	Hopkins and Torres (1989), Lancraft et al. (1991), Geiger et al. (2000)
Electrona carlsbergi	Oceanic, Mesopelagic	Salps, euphausiids, copepods, amphipod; not found in McMurdo Sound	Shreeve et al. (2009)
Gymnoscopelus nicholsi	Oceanic, Mesopelagic	Euphausiids, copepods; not found in McMurdo Sound	Shreeve et al. (2009)
Mollusca			
Squid - Onychoteuthidae			
Kondakovia longimana	Benthopelagic	*E. superba*; diet in McMurdo Sound is not known	Filippova (1972)
Squid- Mastigoteuthidae			
Mastigoteuthis psychrophila	Pelagic	*E. superba*	Kear (1992), Lu and Williams (1994)
Squid- Psychroteuthidae			
Psychroteuthis glacialis	Pelagic	*E. superba*, fish; diet in McMurdo Sound is not known	Kear (1992), Lu and Williams (1993), Collins et al. (2004)

Family and Species	Habitat	Diet	Reference
Octopus - Octopodidae			
Pareledone turqueti	Benthic	Amphipods, fish, polychaetes; diet in McMurdo Sound is not known	Piatkowski et al. (2003)
Pareledone 1 *(albimaculata)*	Benthic	Diet not known	
Pareledone 2 *(aequipapillae)*	Benthic	Diet not known	
Pareledone 3 *(charcoti)*	Benthic	Diet not known	
Pareledone 4 (*turqueti*)	Benthic	Diet not known	
Gastropod - Lamelleriidae			
Marseniopsis mollis	Sediment surface	*C. verrucosa*	Dayton et al. (1974)
Crustacea			
Krill - Euphausiidae			
Euphausia crystallorophias	Pelagic	Phytoplankton, micro and mesozooplankton	O'Brien (1987), Pakhomov et al. (1998)
Euphausia superba	Pelagic	Phytoplankton, zooplankton, algae, diatoms, copepods	O'Brien (1987), Cripps and Hill (1998), Cripps et al. (1999), Haberman et al. (2003)
Isopod - Chaetiliidae			
Glyptonotus antarcticus	Sediment surface	Echinoderms, gastropods, pelecypods, ophiuroids	Dearborn (1967)
Amphipod - Lysianassidae			
Unknown sp. from McMurdo Sound	Pelagic	Scavenger	Slattery and Oliver (1986)
Echinodermata			
Sea star- Odontasteridae			
Odontaster validus	Sediment surface	Detritus, sponges, hydroids, byrozoans, molluscs	Dayton et al. (1974), McClintock (1994)
Sea cucumber- Cucumariidae			
Staurocucumis turqueti	Sediment surface	Diet not known	
Sea urchin - Echinidae			
Sterechinus neumayeri	Sediment surface	Detritus	Dayton et al. (1974)
Other Invertebrates			
Polychaete - Flabelligeridae			
Flabelligera mundata	Sediment surface	Deposit feeder	Clarke (2008)
Nemertean -Cerebratulidae			
Parborlasia corrugatus	Sediment surface	Sponges, diatoms, sea stars, anemones, polychaetes	Dayton et al. (1974), Dayton (1989), Gibson (1983)
Tunicate - Styelidae			
Cnemidocarpa verrucosa	Sediment surface	Suspension feeder	McClintock et al. (1991)

4.2. Materials and methods

4.2.1 Sample collection

Fish and invertebrates from various locations within McMurdo Sound (Figures 4.1 and 4.2) were captured during the austral spring (October-December) in 2006 and 2007 during research projects of Dr. Gretchen Hofmann (University of California at Santa Barbara, CA,

USA), Dr. Art DeVries (University of Illinois, Urbana-Champaign, USA), Dr. Victoria Metcalf (Lincoln University, Christchurch, New Zealand) and Drs. Olav Oftedal and Regina Eisert (Smithsonian Environmental Research Center, Edgewater, MD, USA). Fish were caught using hook and line and following euthanasia were snap-frozen whole in liquid N_2. Invertebrates were collected using baited fish traps set at varying depths (all < 50 m) or by hand while diving and snap-frozen whole. One species of Lysianassid amphipod (possibly *Abyssorchomene* sp.) was collected in McMurdo Sound using seal carcasses as bait, but their ID is still to be confirmed. *Pareledone turqueti* (octopus) were captured in fish traps set at a depth of 450-500 m to the west of Turtle Rock (Figure 4.2). The species ID of this *Pareledone* was confirmed by morphology and genetic barcoding (O. Oftedal, personal communication). Octopods were held at the McMurdo Aquarium for about two weeks until killed by snap freezing. All samples were of whole fish except *D. mawsoni*, for which a sample of dorsal trunk musculature dissected posterior to the pectoral fins was provided. This an appropriate sample for this prey species as Weddell seals do not consume the entire fish but rather remove and consume muscle and viscera (Ainley and Siniff 2009).

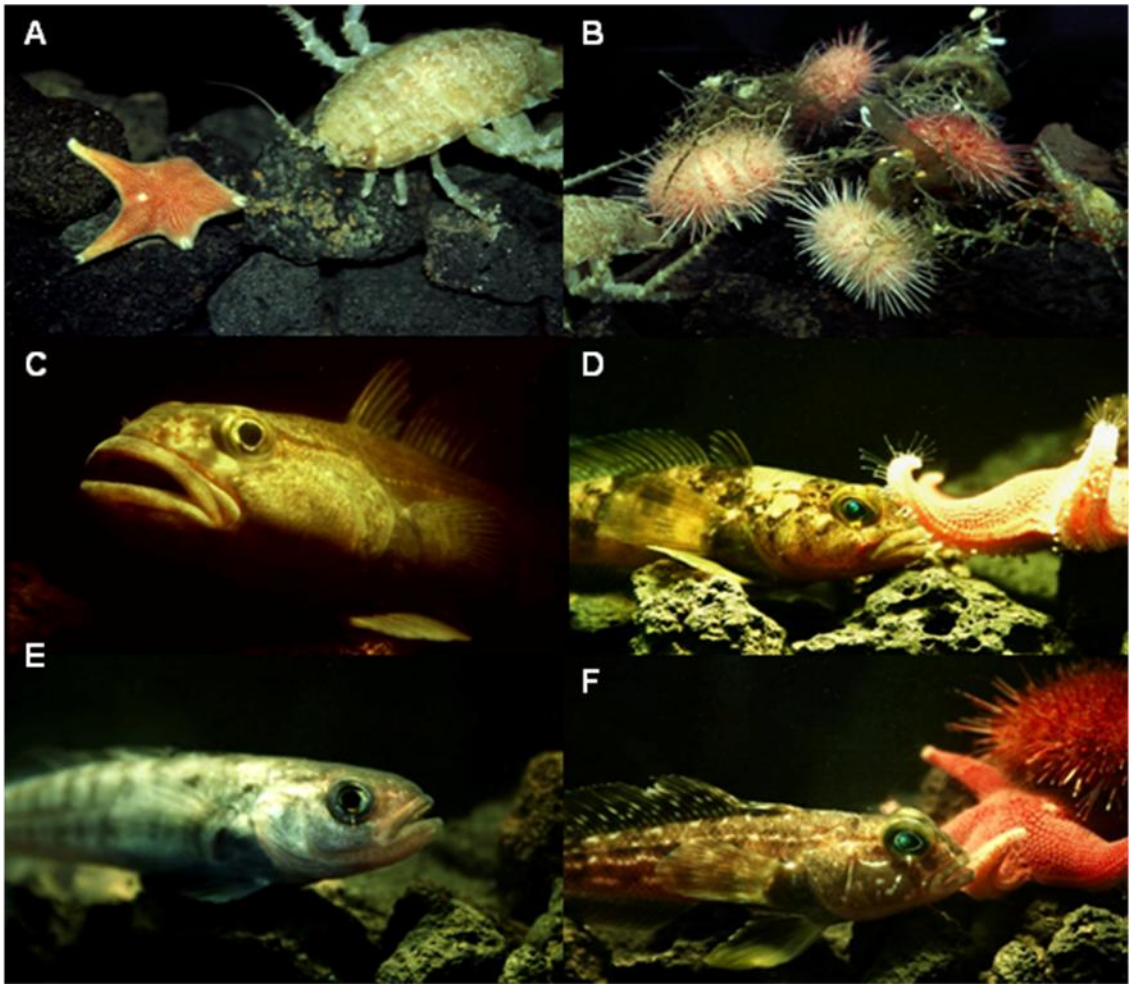


Figure 4.1. Fish and invertebrates collected from various locations around McMurdo Sound in 2006 and 2007. (**A)** *Odontaster validus* and *Glyptonotus antarcticus*, (**B)** *Sterechinus neumayeri*, (**C)** *Trematomus hansoni*, (**D)** *Trematomus bernacchii*, (**E)** *Pagothenia borchgrevinki* and (**F)** *Trematomus pennellii.* (Photos by Victoria Metcalf)

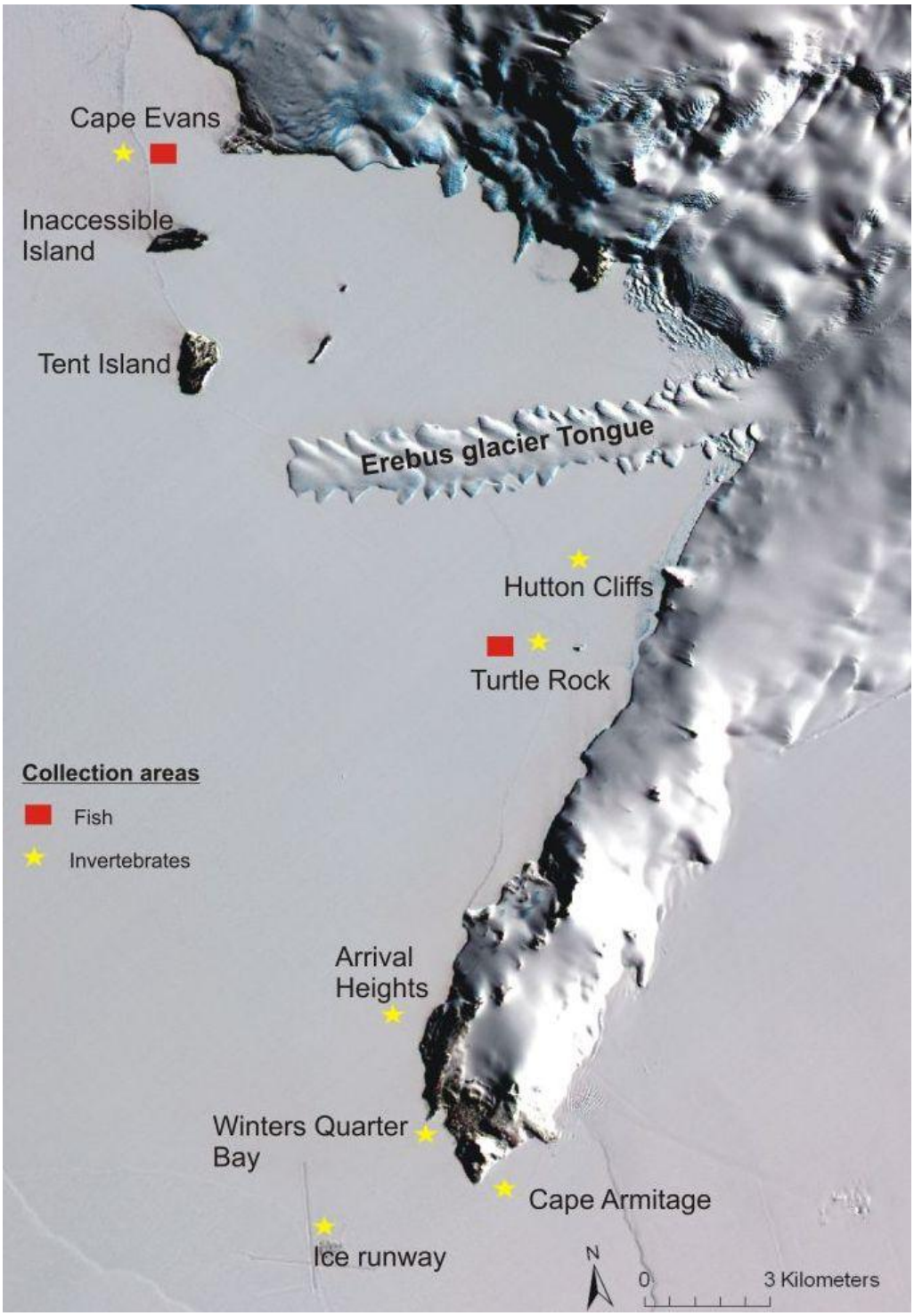


Figure 4.2. Map showing the collection areas of fish (red squares) and invertebrates (yellow stars) from McMurdo Sound.

Dr. Matt Pinkerton (National Institute of Water and Atmospheric Research (NIWA), Wellington, New Zealand) provided a number of fish (Figure 4.3) and cephalopod samples

collected during New Zealand's IPY - Census of Antarctic Marine Life (CAML) survey in the open Ross Sea (between 65º-75ºS and 170ºE-175ºW; Figure 4.4) aboard the RV Tangaroa in the austral summer (February-March) of 2008. Samples were caught in demersal, midwater and pelagic trawls (Hanchett 2008). *Pareledone* species from the Ross Sea were tentatively identified based on morphological features, and are believed to be new as yet unnamed species (Garcia 2010), but molecular genetic data were not available at the time this thesis was written. These species are referred to as *Pareledone* 1 (resembles *P. albimaculata*), 2 (resembles *P. aequipapillae*), 3 (resembles *P. charcoti*) and 4 (resembles *P. turqueti*). All samples were collected in accordance with relevant collection and animal ethics permits issued to the collector in each instance. Details about collection date, site and depth for each sample are provided in Appendix B.

Figure 4.3. Fish collected from various locations in the Ross Sea in 2008. (**A**) *Pleuragramma antarcticum*, (**B**) *Trematomus scotti*, (**C**) *Trematomus lepidorhinus*, (**D**) *Trematomus eulepidotus*, (**E**) *Electrona carlsbergi*, (**F**) *Electrona antarctica*, (**G**) *Gymnoscopelus nicholsi* and (**H**) *Bathylagus antarcticus*. (Photos provided by Peter Marriott, NIWA)

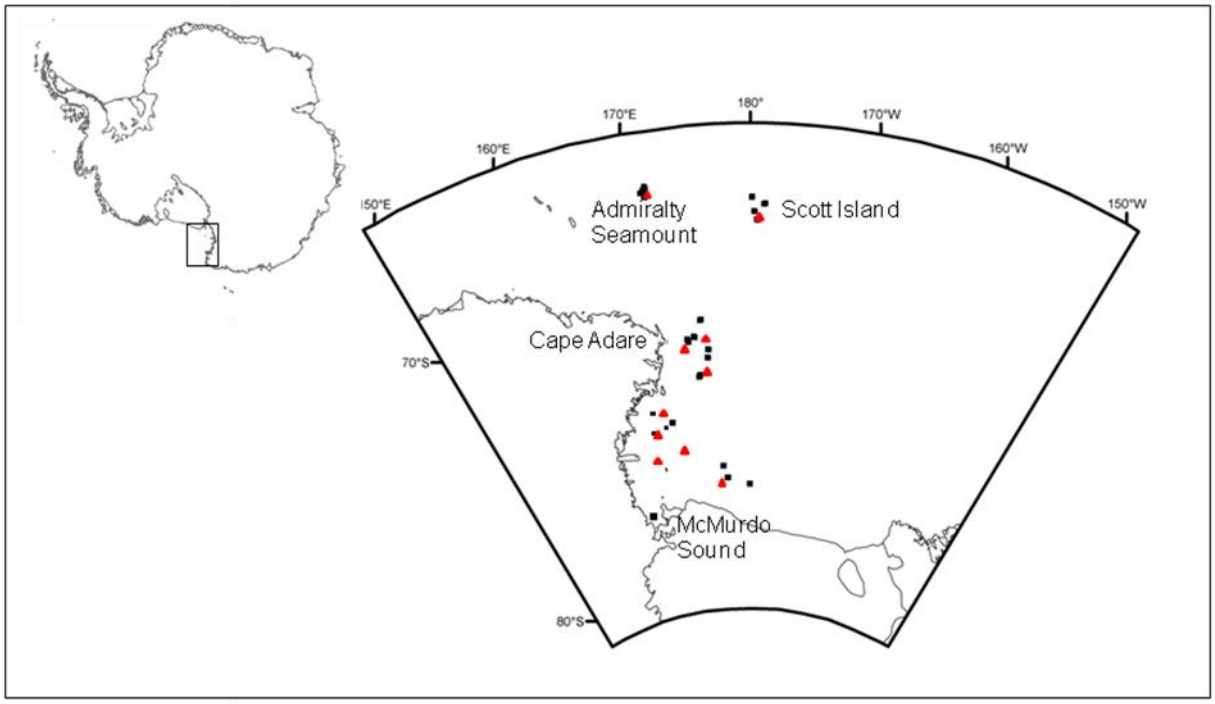


Figure 4.4. Map showing the collection areas of fish (black squares) and cephalopods (red triangles) from the Ross Sea.

4.2.2 Sample preparation and analysis

Fish of <100 gram wet mass (WM) were pooled (n = 2–30 fish, depending on prey size) to obtain sufficient mass for all analyses reported herein as well as all proximate composition assays (Chapter Six) and trace element assays to be published elsewhere. All invertebrates were pooled by species and analysed whole except sea urchins (*Sterechinus*) for which the shell and spines were removed, and cephalopods, for which beaks were removed. Frozen specimens were partially thawed and individuals or pooled specimens were homogenised whole (or after being cut into small pieces) with distilled water (added 1:1 m/m) in a commercial food blender.

Samples were prepared using the procedure outlined in Chapter Two, section 2.2.2 for measuring TMAO and GB using ^{1}H NMR. Briefly, one gram of wet homogenate was weighed into a 10 mL Corning tube and extracted repeatedly (n = 3) with 3 mL aliquots of cold 6% PCA and 1 mL DCM. Samples were vortexed for 30 seconds and then shaken at room temperature for 30 minutes. The polar and lipid layers were separated by centrifugation

at 12,000*g* for 5 minutes. Polar fractions (supernatant) from repeated extractions were pooled into a clean, labelled 10 mL centrifuge tubes and the total volume recorded by mass. For squid and octopus (except *P. turqueti* from McMurdo Sound), only a small amount of freeze-dried material (~50 to 200 mg) was available for analysis, and this was extracted as described for fresh homogenate but the volume of PCA was adjusted for dry material. A 400 µL subsample of fish or invertebrate extract was then analysed by ^{1}H NMR using the method outlined in section Chapter Two, section 2.2.3.

AsB, homarine and DMSP were measured in the same PCA extracts as TMAO and GB but using the LC-MS/MS method outlined in Chapter Three, section 3.3.2. A 25 µL aliquot of PCA extract was added to 500 µL of dilution solvent containing 90% ACN and 10% MeOH and 2 µM each of the internal standards $^{13}C_2$-arsenobetaine, D_4-homarine and D_6-DMSP. Samples were shaken for 5 minutes, centrifuged for 5 minutes at 13,000 rpm, then transferred to 96-well polypropylene microtitre plates and covered until the start of the analysis. Plates were stored at -20 ºC and run within 12 hours using the LC-MS/MS parameters described in Chapter Three, section 3.3.3.

4.2.3 Statistical analysis

Non-metric multidimensional scaling (nMDS) was used to identify biomarker patterns and determine whether these patterns could be used to differentiate between feeding on different types of prey. For nMDS analyses, species were first divided into two broad groups: 1) fish and 2) invertebrates. Secondly, species were divided into seven taxonomic groups: fish, which were grouped by family into 1) nototheniids (n = 15), 2) myctophids (n = 3), and 3) bathylagid (n = 1) and invertebrates, which were grouped into 4) molluscs (cephalopods and gastropod, n = 10), 5) crustaceans (amphipod, isopods, krill, n = 5), 6) echinoderms (sea urchin, sea star, sea cucumber, n = 3) and 7) other (worms and tunicate, n = 3). Lastly, analyses were run with four food groups that represent potential prey of Weddell seals: 1) nototheniids, 2) myctophids, 3) molluscs and 4) crustaceans. nMDS results are presented visually in a two-dimensional (2D) plot or "ordination". The accuracy of the 2D plot is indicated by the stress value (Kruskall's stress formula) in the right-hand corner of the plot. Stress values < 0.1 indicate a good ordination with no prospect of misinterpretation (Clarke 1993). All plots were based on Euclidean distance similarity matrices of normalised data using 50 repetitions. One-way analysis of similarity (ANOSIM) was used to test the null

hypothesis that there were no differences between fish and invertebrates (including cephalopods) and the four food groups in their biomarker composition. SIMPER (similarity of percentages) identifies the biomarkers that contribute most to the dissimilarity between the taxonomic groups. All analyses were performed using the program PRIMER v6 (PRIMER-E Ltd, Plymouth, UK). Theoretical aspects of nMDS, clustering and SIMPER are described by Clarke and Gorley (2006). For individual biomarkers, analysis of variance (ANOVA) was used to compare groups using the statistical package SPSS (PASW version 18.0.0). Tukey's test was used for *ex post facto* comparison (α = 0.05) to determine which groups differed from each other.

Due to differences in concentrations, TMAO and GB results are expressed as mmol kg^{-1} (on a fresh mass basis) while AsB, DMSP and homarine are expressed as µmol kg^{-1} (on a fresh mass basis) with standard error of the mean (SEM) for individually analysed organisms only. Values obtained for freeze-dried cephalopods were converted from dry mass (as analysed) to fresh mass based on % dry matter measured in *P. turqueti* collected from McMurdo Sound (Chapter Six). A value of zero was applied if a biomarker was not detected.

4.3 Results

4.3.1 Biomarkers in prey

Biomarker concentrations in 14 species of Antarctic fish and 19 species of Antarctic invertebrates are reported in Table 4.2. TMAO was present in all species except *Flabelligera mundata* (polychaete worm) and *Parborlasia corrugatus* (nemertean worm), and was highest in fish. TMAO was the dominant biomarker of those measured in all fish species and highest in *D. mawsoni* muscle tissue (162.1 mmol kg^{-1}). GB was present in all species and highest in the octopus *Pareledone* 2 (91.2 mmol kg^{-1}). AsB was present in trace or low amounts in all of the fish species (Table 4.2), but was higher in *Trematomus* species collected from McMurdo Sound compared to fish analysed from the Ross Sea. AsB was highest in the octopus *P. turqueti* (760 µmol kg^{-1}) followed by *Glyptonotus antarcticus* (560 µmol kg^{-1}) collected from McMurdo Sound. In fish, homarine was present in the highest concentrations in *Trematomus* species from McMurdo Sound, up to 1.2 mmol kg^{-1} in *T. pennellii*, but among invertebrates was highest in *P. turqueti* from McMurdo Sound and the Antarctic krill *Euphausia superba* (both about 12 mmol kg^{-1}; Table 4.2) collected from the southeastern Ross Sea. DMSP concentrations were highest in several nototheniid species common in McMurdo Sound

including *T. bernacchii*, *T. hansoni* and *T. pennellii*. DMSP was not detected in any molluscs except *P. turqueti* from McMurdo Sound, but occurred in fairly high concentrations in amphipods, *G. antarcticus*, and the sea cucumber *Staurocucumis turqueti* (Table 4.2).

Table 4.2. Biomarker concentrations on a fresh mass basis measured in fish, cephalopods and invertebrates from the Ross Sea (RS) and McMurdo Sound (MS), Antarctica. Values are expressed as mean ± SEM for individually analyzed fish only. Values for TMAO and GB obtained using ^{1}H NMR and AsB, homarine and DMSP using LC-MS/MS.

Species	*n*	Collection area	Pooled or individual	TMAO (mmol kg^{-1})	GB (mmol kg^{-1})	AsB (µmol kg^{-1})	homarine (µmol kg^{-1})	DMSP (µmol kg^{-1})
Notothenidae								
Dissostichus mawsoni	1	MS	individual[a]	162	4	300	25	333
Lepidonotothen squamifrons	5	RS	pooled	64	4	16	54	ND
Pagothenia borchgrevinki 2006	2	MS	pooled	88	17	113	646	707
Pagothenia borchgrevinki 2006	3	MS	individual	81 ± 1.0	18 ± 3.6	126 ± 28.5	759 ± 166.1	619 ± 122.8
Pagothenia borchgrevinki 2007	2	MS	pooled	89	12	86	384	448
Pagothenia borchgrevinki 2007	4	MS	individual	88 ± 3.4	15 ± 0.7	138 ± 11.4	640 ± 73.0	448 ± 25.9
Pleuragramma antarcticum	15	RS	pooled	79	4	22	ND	ND
Trematomus bernacchii 2006	6	MS	pooled	70	17	71	919	715
Trematomus bernacchii 2007	7	MS	pooled	70.0	10	95	686	668
Trematomus bernacchii 2007	3	MS	individual	69 ± 1.4	9 ± 2.9	137 ± 25.9	759 ± 228.1	703 ± 159.2
Trematomus eulepidotus	5	RS	pooled	80	1	134	208	106
Trematomus hansoni	7	MS	individual	70 ± 2.7	8 ± 2.0	199 ± 33.8	259 ± 50.8	187 ± 105.7
Trematomus lepidorhinus	5	RS	pooled	134	7	21	174	ND
Trematomus pennellii	2	MS	pooled	74	11	68	1,264	1,249
Trematomus scotti	5	RS	pooled	72	9	105	267	ND
Bathylagidae								
Bathylagus antarcticus	5	RS	pooled	76	4	9	141	ND
Myctophidae								
Electrona antarctica	30	RS	Pooled	49	6	12	84	28
Electrona carlsbergi	15	RS	Pooled	34	7	11	56	37
Gymnoscopelus nicholsi	5	RS	Pooled	52	8	ND	82	ND
Mollusca								
Kondakovia longimana (75)	2	RS	pooled	4	11	2	379	ND
Kondakovia longimana (74)	1	RS	individual	13	13	16	2,022	ND
Mastigoteuthis psychrophila	1	RS	individual	8	6	7	1,230	ND
Psychroteuthis glacialis	4	RS	pooled	28	32	8	4,250	ND
Pareledone turqueti	4	MS	pooled	22	57	760	11,966	19
Pareledone 1 (albimaculata)	3	RS	pooled	7	40	323	8,987	ND
Pareledone 2 (aequipapillae)	2	RS	pooled	20	91	188	2,792	ND
Pareledone 3 *(charcoti)*	3	RS	pooled	5	24	67	4,338	ND
Pareledone 4 *(turqueti)*	7	RS	pooled	3	46	162	3,621	ND

Species	*n*	Collection area	Pooled or individual	TMAO (mmol kg^{-1})	GB (mmol kg^{-1})	AsB (µmol kg^{-1})	homarine (µmol kg^{-1})	DMSP (µmol kg^{-1})
Marseniopsis mollis	8	MS	pooled	3	12	14	1,487	ND
Crustacea								
Lysianassid amphipod	~400	MS	pooled	6	20	48	ND	645
Euphausia crystallorophias (33)	~100	RS	pooled	7	3	ND	9,246	ND
Euphausia crystallorophias (78)	~100	RS	pooled	34	18	13	1,321	ND
Euphausia superba	~100	RS	pooled	63	24	9	11,956	19
Glyptonotus antarcticus	3	MS	pooled	24	17	560	2,563	436
Echinodermata								
Odontaster validus	6	MS	pooled	2	3	77	295	ND
Staurocucumis turqueti	4	MS	pooled	1	11	11	3,807	380
Sterechinus neumayeri	7	MS	pooled	2	2	6	166	ND
Other Invertebrates								
Cnemidocarpa verrucosa	4	MS	pooled	2	33	5	ND	ND
Flabelligera mundata	3	MS	pooled	ND	2	11	33	34
Parborlasia corrugatus	4	MS	pooled	ND	1	23	4,724	25

[a] Biomarker concentrations for *Dissostichus mawsoni* are for a fillet of white muscle. ND = not detected.

4.3.2 Comparison of biomarker composition

Fish and invertebrates differed significantly in TMAO (ANOVA, $F_{1,38} = 83.037$, $P < 0.001$), GB (ANOVA, $F_{1,38} = 5.956$, $P < 0.020$) homarine (ANOVA, $F_{1,38} = 14.449$, $P < 0.001$) and DMSP (ANOVA, $F_{1,38} = 6.932$, $P = 0.012$) concentrations. There was no significant difference in AsB (ANOVA, $F_{1,38} = 0.241$, $P = 0.627$) concentrations between the two groups. nMDS ordination shows that fish and invertebrates cluster separately from each other (Figure 4.5, stress coefficient = 0.1). Application of the ANOSIM test also revealed statistically significant differences in the biomarker composition between the two groups (global R = 0.34, $P < 0.001$). SIMPER tests revealed that DMSP (64%) contributed most to the dissimilarity amongst different species of fish, followed by TMAO (22%). Amongst invertebrates, homarine, GB and AsB contributed equally (30%) to dissimilarity between the different taxa.

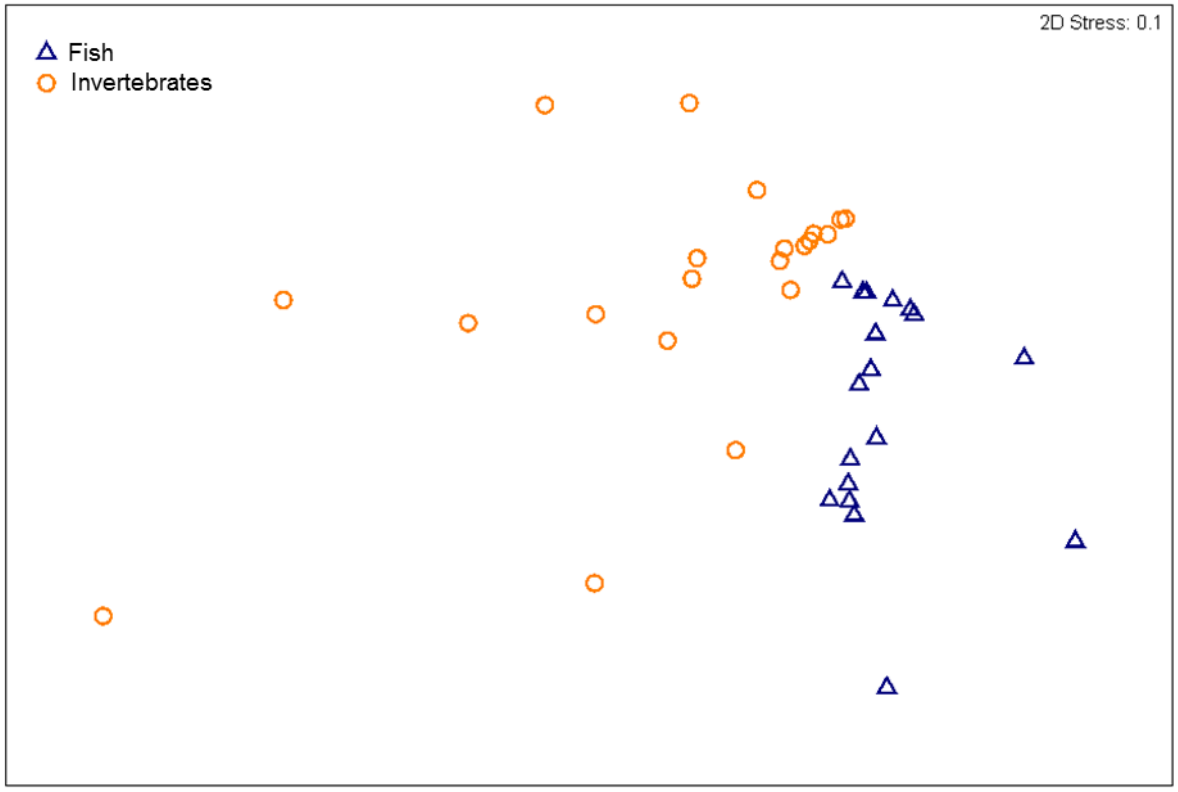


Figure 4.5. nMDS plot showing the degree of similarity in biomarker composition between fish (blue triangles) and invertebrates (orange circles).

nMDS ordination also shows that species in most taxonomic groups are clustered (Figure 4.6, stress coefficient = 0.1). However, individual species vary in their biomarker composition. For example, *D. mawsoni* muscle tissue is not similar in biomarker composition to any other whole fish or nototheniid species analysed whole. For this species only a fillet of white muscle was analysed, but *Dissostichus* contained the highest concentrations of TMAO (162 mmol kg^{-1}, Table 4.2) which likely explains its dissimilarity to the rest of the fish.

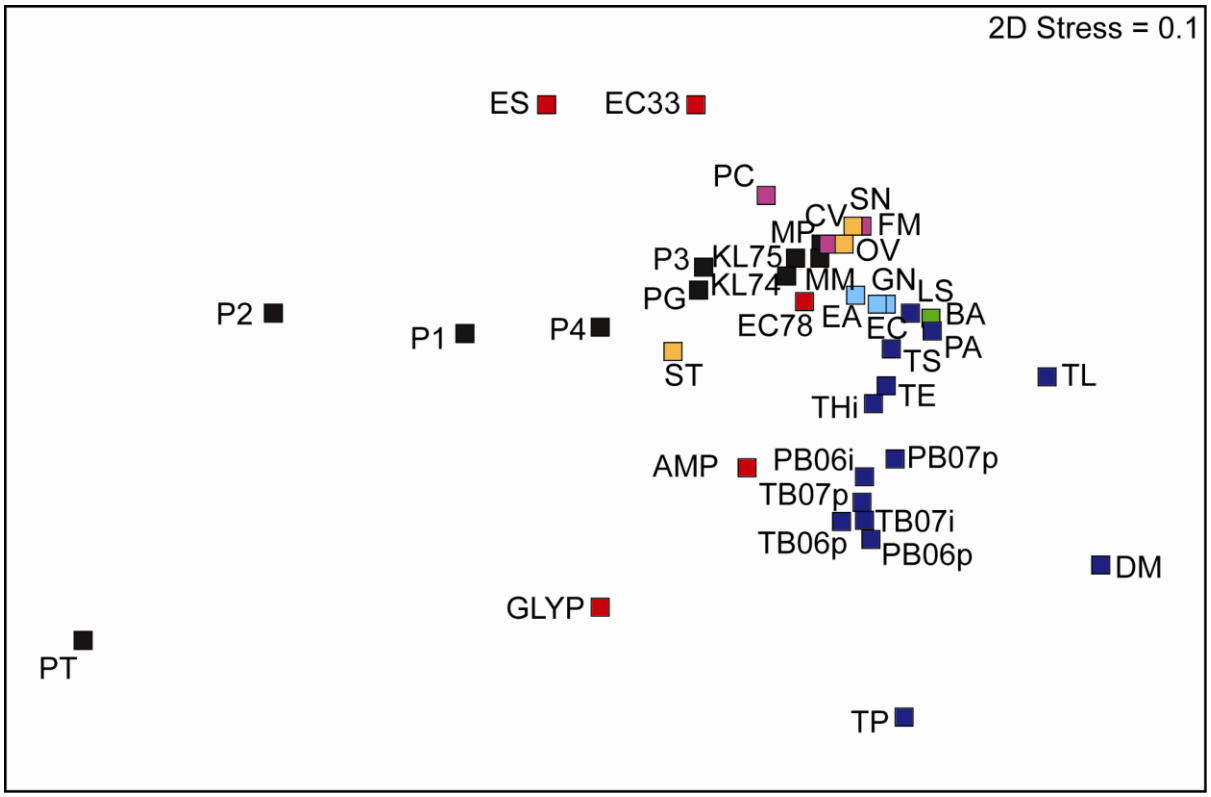


Figure 4.6. nMDS plot showing the degree of similarity in biomarker composition between taxa. Stress value (2D) is given in the top right hand corner of the plot. Biomarker concentrations reported in Table 4.2. **Notothenids (dark blue squares)** DM=*Dissostichus mawsoni*, LS=*Lepidonotothen squamifrons*, PB =*Pagothenia borchgrevinki*, PA=*Pleuragramma antarcticum*, TB=*Trematomus bernacchii*, TE=*Trematomus eulepidotus*, THi=*Trematomus hansoni*, TL=*Trematomus lepidorhinus*, TP=*Trematomus pennellii*, TS=*Trematomus scotti*. **Myctophids (light blue squares)** EA=*Electrona antarctica*, EC=*Electrona carlsbergi*, GN=*Gymnoscopelus nicholsi*. **Bathylagid (green squares)** BA=*Bathylagus antarcticus*. **Molluscs (black squares)** KL74 and 75=*Kondakovia longimana*, MM=*Marseniopsis mollis*, MP=*Mastigoteuthis psychrophila*, P1=*Pareledone* cf. *albimaculata*, P2=*Pareledone* cf. *aequipapillae*, P3=*Pareledone* cf. *charcoti*, P4= *Pareledone* cf. *turqueti*, PG=*Pyschroteuthis glacialis*, PT(MS)=*Pareledone turqueti* McMurdo Sound. **Crustaceans (red squares)** AMP=amphipod, EC33 and 78=*Euphausia crystallorophias*, ES=*Euphausia superba*, GLYP=*Glyptonotus antarcticus*. **Echinoderms (orange squares)** OV=*Odontaster validus*, ST=*Staurocucumis turqueti*, SN=*Sterechinus neumayeri*. **Other invertebrates (purple squares)** CV=*Cnemodicarpa verrucosa*, PC=*Parborlasia corrugatus*, FM=*Flabelligera mundata*.

Pagothenia borchgrevinki and *T. bernacchii*, common species in McMurdo Sound, are closely clustered while fish species collected from the open Ross Sea (with the exception of *Trematomus lepidorhinus*) are more closely clustered. *Trematomus pennellii* (TP) is clustered separately from the rest of the nototheniids collected in McMurdo Sound (Figure 4.6). For this species two specimens were pooled together and *T. pennellii* contained the highest concentrations of DMSP and homarine (Table 4.2) which likely explains its dissimilarity to the rest of the fish. There was no apparent difference in biomarker composition between individually analysed and pooled samples for *P. borchgrevinki* or *T. bernacchii* (Figure 4.6). Interestingly, *Pleuragramma antarcticum*, a schooling pelagic fish which was collected from mid-water trawls, is most similar in biomarker composition to *Bathylagus antarcticus*, a deep water fish that was caught at depths over 1,000 m (Hanchett 2008). SIMPER tests revealed that DMSP (64%) contributed most to the dissimilarity amongst different species of nototheniids, followed by TMAO (22%).

Glyptonotus antarcticus (giant isopod) is clustered separately from the rest of the crustaceans (Figure 4.6) and this is probably due to the large amounts of AsB and homarine that it contains. SIMPER tests revealed that homarine (41%) and AsB (36%) did indeed contribute most to the dissimilarity amongst crustaceans. It is also surprising that *Euphausia crystallorophias* (EC 33) collected from a midwater trawl near Terra Nova Bay is more similar to *E. superba* (ES) caught at deeper depths (800 m) than to *E. crystallorophias* (EC 78) which was also collected from a midwater trawl but in a different section of the Ross Sea.

Amongst the molluscs, AsB and GB (38 and 39%) contributed most to the dissimilarity between the different types of octopus and squid. *Pareledone turqueti* (PT) is separated from the rest of the octopus species (Figure 4.6) while *Kondakovia longimana* (KL 74 and 75) and *Mastigoteuthis psychrophilia* (MP) are more similar to species of other taxa, including the gastropod *M. mollis* (MM, Figure 4.6). Even though they were sampled at different locations, *K. longimana* collected near the Admiralty seamounts (KL75) did not differ in biomarker composition from *K. longimana* (KL74) collected near Scott Island (Figure 4.6). *Pareledone turqueti* collected from McMurdo Sound has a vastly different biomarker composition to the *Pareledone* specimens collected in the Ross Sea (*Pareledone* 4, P4) that were morphologically similar to *P. turqueti*, which is consistent with the hypothesis (Garcia 2010) that these latter octopods represent new species.

In order to relate the biomarker data to lactating Weddell seals, the data set was re-analysed after restricting the prey to four food groups (nototheniids, myctophids, molluscs and crustaceans) known to be consumed by Weddell seals, as other invertebrates are not believed to be potential diet items other than as secondary ingestion, *i.e.*, gastrointestinal contents of target prey (Dearborn 1965, Castellini et al. 1992, Casaux et al. 1997, Burns et al. 1998, Ainley and Siniff 2009, Casaux et al. 2009). Similar trends were observed in this subset as for the entire set of samples in that *P. borchgrevinki* and *T. bernacchii* are still clustered closely together, and *D. mawsoni* muscle tissue is still separated from the rest of the fish as is *T. pennellii* (Figure 4.7). Octopus, though widely distributed in Figure 4.7, are still clustered separately from squid, crustaceans and fish.

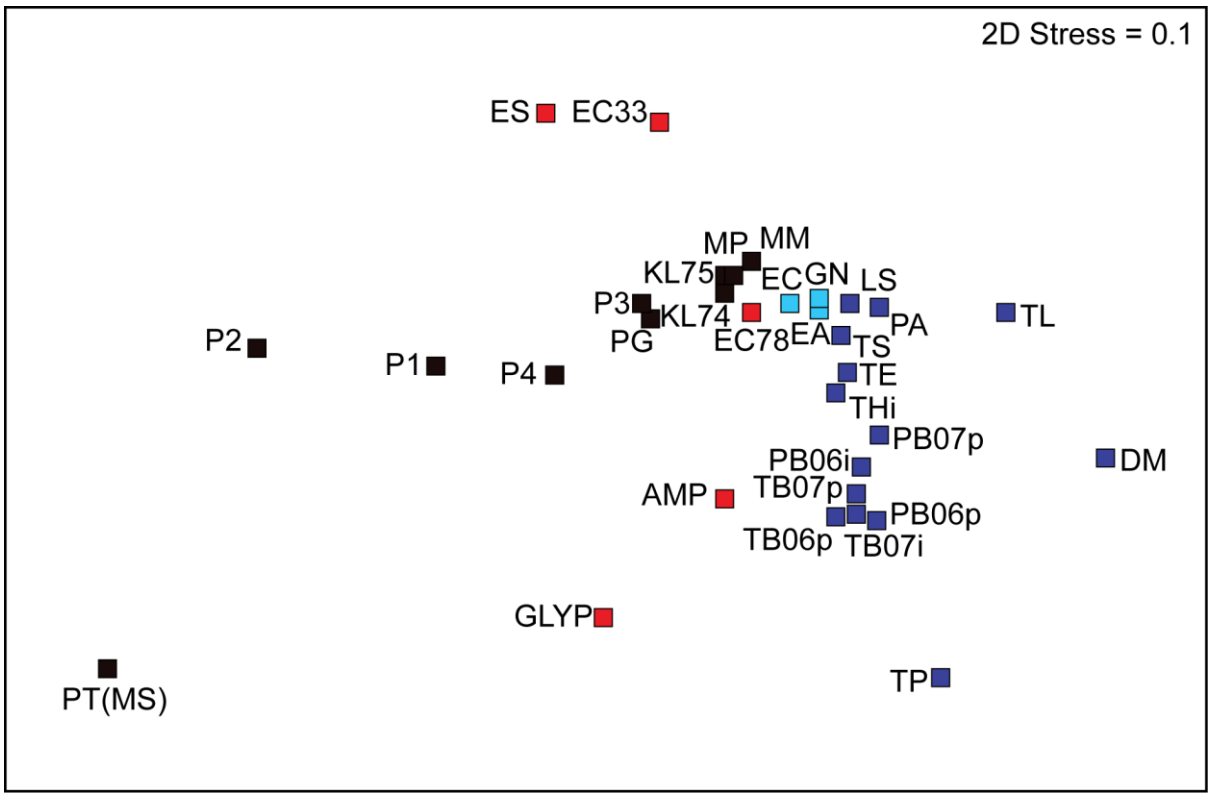


Figure 4.7. Degree of similarity in the biomarker concentration in known and potential prey species of Weddell seals using nMDS. Species abbreviations are provided in Figure 4.6.

Application of the ANOSIM test revealed statistically significant differences in the biomarker composition between the four food groups (global R = 0.38, $P < 0.001$). TMAO differed amongst the groups (ANOVA, $F_{3,31} = 25.527$, $P < 0.001$) with a significant difference between nototheniids and the three other food groups (Tukey's test, all $P < 0.001$, Figure 4.8). There were no differences amongst crustaceans, molluscs and myctophids. Glycine betaine differed amongst the prey groups (ANOVA, $F_{3,31} = 4.881$, $P = 0.007$) with a significant difference between nototheniids and crustaceans (Tukey's test, $P = 0.007$). Homarine also differed amongst the prey groups (ANOVA, $F_{3,31} = 5.278$, $P = 0.005$), with a significant difference between crustaceans and nototheniids (Tukey's test, $P = 0.025$) and molluscs and nototheniids (Tukey's test, $P = 0.024$, Figure 4.8). There were no significant differences in homarine concentrations between myctophids and nototheniids, or between myctophids, molluscs and crustaceans. DMSP differed amongst the prey groups (ANOVA, $F_{3,31} = 4.476$, $P = 0.011$) with a significant difference between nototheniids and molluscs (Tukey's test, $P = 0.009$). AsB concentrations were not significantly different amongst the four food groups (ANOVA, $F_{3,31} = 0.589$, $P = 0.627$, Figure 4.8).

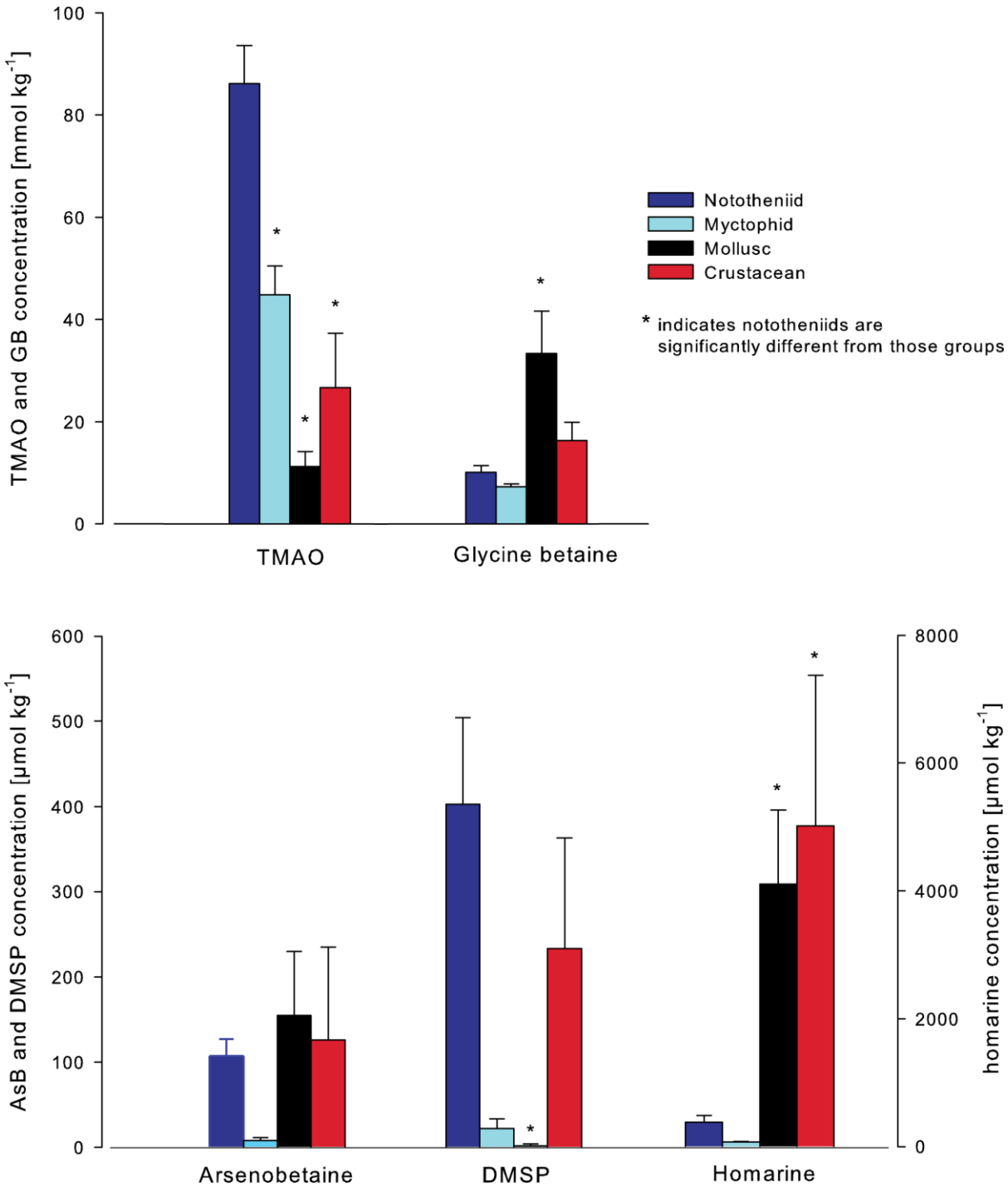


Figure 4.8. Distribution of biomarkers amongst the four food groups. Concentrations are means with standard error bars. Note that homarine concentrations are provided on the right y-axis due to differences in concentration from AsB and DMSP. Number of samples analyzed in each group n = 15 nototheniids, n = 3 myctophids, n = 10 molluscs and n = 5 crustaceans.

4.4 Discussion

The variation in biomarker composition amongst various Antarctic species confirms that marine species tend to accumulate different compounds (Carr et al. 1996). For example, TMAO was not detected in either nemertean or polychaete worms in this study; however none of the other biomarkers measured featured predominantly in their extracts (Table 4.2). TMAO and other methylamines are typically better protein stabilisers than other osmolytes (Yancey 1994), which suggests that nemertean and polychaete worms may use a different class of osmolytes in order to survive in sub-zero water temperatures. This is similar to previous studies in vestimentiferan worms living in hydrothermal vents where TMAO was not detected, even though it is generally abundant in deep-sea organisms, and sulfur compounds (specifically sulfonates) such as taurine and hypotaurine were the dominant osmolytes (Yin et al. 2000).

Previously, increased levels of TMAO and AsB in lactating Weddell seal plasma have provided evidence of feeding (Eisert et al. 2005), but the results from this study show that TMAO and AsB alone do not provide sufficient information for distinguishing between prey types consumed by seals. AsB has been shown to magnify through the food web (Foster et al. 2006); however, this was not the case in this study. It would be difficult to predict feeding preferences based solely on AsB because organisms from each phylum contained AsB and there were no significant differences in AsB concentrations between the four food groups identified in this study. The dominant source of AsB in fish and molluscs is likely from the diet as fish in general do not appear to synthesise AsB (Francesconi and Edmonds 1994). TMAO was present in almost all species, but was highest in certain Antarctic fish (Table 4.2) and therefore would be a good biomarker for detecting feeding if a predator's diet was based solely on fish. At this stage it is not possible to distinguish what species of fish Weddell seals may be feeding on given the presence of TMAO in all of the species analysed.

Homarine and DMSP appear to be two suitable biomarkers for distinguishing between different food groups of Weddell seals because their occurrence was found to vary considerably between species/taxa. Homarine was not detected in *Pleuragramma antarcticum*, was measured in *D. mawsoni* at concentrations of 25 μmol kg^{-1} (Table 4.2), and detected at over 1,000 μmol kg^{-1} in some nototheniids from McMurdo Sound (Table 4.2). The source of homarine in fish is most likely from the diet as stomach contents from *D.*

mawsoni indicate this species consumes both squid and octopus in the Ross Sea (Bury et al. 2008), which are expected to contain high concentrations of homarine (Table 4.2); squid has been found to contain high levels of homarine in previous studies (Carr et al. 1996). Homarine concentrations were highest in *Pareledone* spp. (especially *P. turqueti*, 12 mmol kg^{-1}). Octopus may be an important component of Weddell seal diets in McMurdo Sound based on stomach content analysis (Dearborn 1965) and faecal diet analysis conducted elsewhere in the Antarctic (Casaux et al. 1997, Lake et al. 2003, Casaux et al. 2009). All of the squid species analysed in this study have been identified previously in Weddell seal diets from McMurdo Sound (Castellini et al. 1992, Burns et al. 1998). Concentrations of GB were also twenty times higher in some cephalopod species (Table 4.2) compared to *P. antarcticum* and *D. mawsoni*. Thus, the detection of homarine and elevated GB levels in seal blood may be a suitable way for distinguishing cephalopods from these fish in Weddell seal diets.

DMSP was not detected in *P. antarcticum* but was found in concentrations up to 1,000 µmol kg^{-1} in *T. pennellii*, *T. hansoni*, and *T. bernacchii* which are three of the more abundant fish in McMurdo Sound and play an important role in the benthic food web. DMSP was also detected in the cryopelagic species *P. borchgrevinki*. The phytoplankton *Phaeocystis antarctica* provides the primary source of DMSP for grazing zooplankton in McMurdo Sound and the Ross Sea during the summer (Elliott et al. 2009). The pteropod *Limacina helicina,* an abundant pteropod that grazes on these blooms, forms part of the diet of *P. bochgrevinki*, *T. bernacchii* and *T. hansoni* (Foster and Montgomery 1993, Vacchi et al. 1994) and most likely provides a trophic pathway for DMSP in the pelagic food web. This was evident in individually analysed *T. hansoni* where DMSP was only detected in three out of seven individuals. Given that Weddell seals in McMurdo Sound have often been considered to feed predominantly on *P. antarcticum* ((Burns et al. 1998) but see Ainley and Siniff 2009) the biomarker method may be an important means for detecting other fish species in their diet.

The biomarkers that were measured not only come from different prey species, but they will have different biological half-lives when ingested by seals. This may be useful for determining recent food intake *versus* feeding over longer periods of time. For example, while GB is synthesised and metabolised in mammals (Lever and Slow 2010), a dose-dependent increase in blood concentrations of GB can be expected when an animal is feeding. GB is metabolised by BHMT and the increase following feeding is short-lived in

humans (Atkinson et al. 2008) even though GB, unlike all of the other betaines measured in this study, is not excreted in the urine (Atkinson et al. 2009). DMSP is especially rapidly metabolised by BHMT and would be expected to have a short biological half-life in seals (Slow et al. 2004). AsB enters tissues as a GB analogue but is not a substrate for BHMT (Lee et al. 2004). AsB is a substrate for BGT-1 (betaine/GABA transporter) and probably other betaine transporters (Randall et al. 1996), so it enters tissues but is not metabolised which accounts for its relatively long half-life (Eisert et al. 2005). TMAO is excreted within twenty-four hours in rats and humans (Zhang et al. 1999) and was found previously to clear faster from circulation than AsB in seal plasma (Eisert et al. 2005). Homarine is not known to be metabolised by mammals and unfortunately there are no data at present on its clearance rate.

Only two species have been identified as primary prey for Weddell seals in McMurdo Sound, *P. antarcticum* from early studies on hard part analysis from stomach contents (Dearborn 1965), faeces and stable isotopes (Castellini et al. 1992, Burns et al. 1998) and *D. mawsoni* from observational evidence (Calhaem and Christoffel 1969, Kim et al. 2005, Ponganis and Stockard 2007) and video cameras (Davis et al. 1999). Minor prey items include *P. borchgrevinki*, *T. bernacchii* and octopus (Dearborn 1965, Green and Burton 1987, Davis et al. 1999). No diet studies have been carried out in this region since the combined faecal and stable isotope study by Burns and colleagues between 1989 and 1994 (Burns et al. 1998). It is possible that Weddell seal diets may have changed since this period, or vary seasonally according to relative prey abundance. Additionally, there is now more conclusive evidence that a portion of females initiate feeding in late lactation (Hindell et al. 2002, Eisert et al. 2005, Wheatley et al. 2008). However, the majority of feeding has been inferred by dive activity (Testa et al. 1989, Hindell et al. 2002, Sato et al. 2002, Sato et al. 2003). These methods cannot confirm underwater prey encounters; biomarkers can therefore provide independent confirmation of feeding when used in conjunction with 2- and 3 dimensional TDRs. As well, a positive identification from biomarkers (*e.g.*, finding a taxon-specific compound such as homarine) is more conclusive, and more specific, than inferring mean diet trophic level from stable isotopes or interpreting fatty acid patterns that integrate many different prey types over a longer time scale of weeks to months.

The purpose of this study was to assess whether prey differ sufficiently in biomarkers and whether TMAO, AsB, GB, homarine and DMSP can be used for determining if a predator is feeding on different prey items. The biomarker data compiled here is composed of thirty-

three Antarctic species from McMurdo Sound and various locations within the Ross Sea. However, this database does not include *T. newnesi*, *T. nicolai* or dragon-fish (Bathydraconidae, such as *Gymnodraco acuticeps*), nor does it include any species of mysids, copepods or pteropods, all potential or known prey species of Weddell seals (Dearborn 1965, Green and Burton 1987, Burns et al. 1998). These species were not analysed due to a lack of material. Nonetheless, they may be potential prey items because either they have been previously caught in McMurdo Sound or are food for other species. The analysis of these species, as well as expanding coverage to include plunderfishes (Artedidraconidae) and eelpouts (Zoarcidae), will improve the quality of the database and provide us with a better understanding of how biomarkers accumulate through the food web.

Chapter Five

New insights into the foraging behaviour of lactating Weddell seals through the use of dietary biomarkers and time depth recorders

Weddell seal underneath the sea ice near Turtle Rock, McMurdo Sound. Photo provided by N. Huerta, Project SCINI.

5.1 Introduction

The Weddell seal is well adapted to life on the Antarctic fast ice, occupying high latitude areas year round. Weddell seals have been studied more than any other Antarctic seal because most Weddell seals return to their natal colonies on the fast ice to breed and give birth, and are easily accessible from research stations (*e.g.*, McMurdo and Scott Base). In Erebus Bay, individual seals have been tagged for several decades, providing researchers with unique opportunities to study the breeding biology and reproductive success of known-age animals (Testa 1986, Hastings and Testa 1998, Cameron et al. 2007).

Despite extensive studies on Weddell seals in McMurdo Sound since the 1960s, uncertainty still remains regarding female foraging habits during the lactation period. This is mainly because female Weddell seals are considered to be capital breeders. Energy stores that females have accumulated prior to parturition should be able to support offspring throughout the entire breeding season. Weddell seals are one of the largest phocids with a long lactation period (6-8 weeks, Kaufman et al. 1975). Based on their large body size and large relative mass loss of ≥ 40% of postpartum body mass (Eisert and Oftedal 2009), it has been assumed that Weddell seals fast or feed to a neglible extent during the lactation period. However, years in which many pups are weaned early may indicate food limitations (Wheatley et al. 2006). Annual fast ice persists throughout most of the spring and summer in McMurdo Sound, reducing productivity and food availability for Weddell seals.

Foraging by lactating females has mainly been inferred by telemetry studies using 2D time-depth recorders (Testa et al. 1989, Sato et al. 2002, Sato et al. 2003). These data loggers are attached to the animal's pelage and record the depth to which the animal is diving. Dive profiles of depth *versus* time can then be produced, and information about dive depth, duration and speed of travel can be calculated (Schreer and Testa 1995, 1996). However, interpreting this information can be challenging. Statistical analyses can only be used to classify patterns and the purpose behind a given dive behaviour needs to be confirmed by independent observations. The use of 3D dive recorders that measure distance and direction in addition to depth allows for the determination of where and how diving occurred in relation to local bathymetric features (Hindell et al. 2002, Mitani et al. 2003). These studies have provided researchers with a better insight into Weddell seal movements beneath the fast ice and seals' use of the water column. However, the main limitation of 2D and 3D dive

profile analysis is that underwater prey encounters cannot be detected. From TDR data alone, it is not possible to determine whether a feeding event has occurred. Animal-borne video cameras have proved useful for investigating hunting strategies and documenting prey encounters of Weddell seals (Davis et al. 1999, Fuiman et al. 2003, Mitani et al. 2004), providing details that help classify dives as foraging dives and the types of foraging strategies employed by Weddell seals (Davis et al. 2003). However, these studies are also bound by limitations. In studies by Davis et al. (1999) and Fuiman et al. (2007), Weddell seals were restricted to diving from an isolated hole away from breeding colonies, and foraging took place over deeper water than present at most breeding colonies. These studies were also conducted on males and non-lactating females. Therefore, the foraging strategies employed by these seals may not be representative of the foraging tactics employed by lactating females within breeding colonies.

Dietary analyses based on examination of hard parts in faeces are not always a convenient option to study foraging during lactation because it is difficult to assign faeces to known animals in large breeding colonies where up to 250 individuals may aggregate (Stirling 1969). Recently developed methods of diet analysis such as fatty acids and stable isotopes are increasingly being used to investigate the feeding habits of marine mammals. At present, only Zhao et al. (2004) and Burns et al. (1998) have utilised stable isotopes in Weddell seal feeding studies but these were not conducted on lactating females. Wheatley et al. (2007) examined fatty acids in blubber samples collected from females during early and late lactation, but found variability in fatty acid mobilisation which precluded conclusive dietary predictions. These methods have their advantages, but both integrate food consumption over periods of weeks to months; therefore, they are not particularly useful for monitoring recent food intake.

Eisert et al. (2005) identified two biomarkers that occur in marine fish and invertebrates, TMAO and AsB, which act as indicators of food intake in marine predators. These compounds produce a biochemical signature in mammalian body fluids such as plasma if an animal is feeding. Dietary biomarkers reflect food intake over short periods, usually within a time scale of hours to days, and provide information on recent feeding prior to excretion (Yancey et al. 1982, Edmonds and Francesconi 1987, Lehmann et al. 2001). Currently the biomarker method can tell us whether a female is feeding or fasting, but not what prey was consumed. The biomarker method developed by Eisert et al. (2005) was expanded upon by

measuring additional biomarkers such as DMSP, GB and homarine in a number of different prey and potential prey items of Weddell seals in Chapter Four in order to determine whether different prey items contained characteristic/specific biomarker patterns.

The aim of this study was to determine the onset and prevalence of foraging in female Weddell seals and to determine what taxa they were feeding on by a combination of dietary biomarkers and 2D TDR data. Concentrations of TMAO, GB, AsB, homarine and DMSP were measured in female plasma throughout the lactation period using LC-MS/MS. TDRs were used to monitor dive activity over the same period. Both approaches have some limitations when used independently. However, their simultaneous use provides a powerful tool to study diet composition and diet variability within individuals over time.

5.2 Methods

5.2.1 Study site and sample collection

This study was conducted on lactating female Weddell seals at the Hutton Cliffs seal colony (77°51'S, 166°45'E) in McMurdo Sound during the austral summer of 2006 (October to December). The lactation stage of study animals was ascertained by noting pup birth dates during daily surveys. Lactation in Weddell seals was divided into three stages, early lactation (EL, 0 to 13 days postpartum [dpp]), mid-lactation (ML, 14 to 26 dpp) and late lactation (LL, $\geq$ 27 dpp).

Adult females were captured at 2–6 day intervals from 2–3 dpp until 30–37 dpp. Females were captured by head-bagging (Stirling 1966) and blood samples were collected into heparinised containers by venipuncture from the extradural vein. Blood samples were placed on crushed ice and centrifuged to obtain plasma within two hours of collection. Plasma was frozen immediately at the field camp on dry ice and stored at -80 ºC in the laboratory until analysis.

2D TDRs (Mk9 archival tags, Wildlife computers, Redmond, WA, USA) were applied between 2 and 3 dpp (6 dpp in one seal) and remained on seals until 29–37 dpp. TDRs were first attached to mesh mats using hose clamps, and then the mesh mats were glued using a five-minute epoxy resin to the dorsal surface of each seal. TDRs were set to sample at ten-

second intervals (when wet) and to a maximum depth of 1,000 meters. Details about TDR deployment and collection date are provided in Table 5.1.

Table 5.1. Time depth recorder (TDR) deployment and recovery details for each female studied over the lactation period in 2006.

Female ID	Age	Mass [kg] Start lactation[a]	Pup DOB	TDR deployment [date]	Pup age [days] TDR deployment	TDR recovery [date]	Pup age [days] TDR recovery	Total days monitored	Mass [kg] End lactation[a]
40	21	458 (3)	24-Oct-2006	26-Oct-06 10:44:00	2.4	1-Dec-06 18:42:00	38.7	36.33	286 (38)
105	10	460 (2)	26-Oct-2006	28-Oct-06 13:44:00	2.5	4-Dec-06 19:43:00	39.8	37.25	282 (38)
115	10	381 (2)	24-Oct-2006	26-Oct-06 10:56:00	2.4	2-Dec-06 22:40:00	39.9	37.49	224 (39)
265	8	449 (3)	24-Oct-2006	26-Oct-06 10:50:00	2.4	1-Dec-06 18:48:00	38.7	36.33	297 (38)
479	13	426 (2)	23-Oct-2006	25-Oct-06 11:27:00	2.4	5-Jan-07 18:06:00	24.7	22.29	302 (24)[b]
515	12	457 (2)	27-Oct-2006	28-Oct-06 13:44:00	1.5	4-Dec-06 19:41:00	38.8	37.25	262 (37)
622	9	418 (2)	27-Oct-2006	TDR not recovered					301(25)
819	16	408 (2)	22-Oct-2006	24-Oct-06 16:18:00	2.6	29-Nov-06 19:02:00	38.7	36.11	252 (38)
946	15	481 (2)	24-Oct-2006	30-Oct-06 15:10:00	6.6[c]	2-Dec-06 22:44:00	39.9	33.32	301 (39)
1043	8	343 (2)	28-Oct-2006	30-Oct-06 15:03:00	2.6	28-Nov-06 19:34:00	31.8	29.19	221 (31)
5746	24	450 (2)	23-Oct-2006	25-Oct-06 11:23:00	2.4	29-Nov-06 18:53:00	37.7	35.31	283 (37)
5891	14	464 (2)	26-Oct-2006	TDR not recovered					346(24)
Overall mean	11	432			2.8		36.8	34	271

[a] Number in parentheses indicates day post partum each seal was weighed. [b] Female 479 was not weighed or sampled after day 24. [c] Seal caught for blood sampling at 2.6 dpp.

5.2.2 Sample preparation

Seal plasma was analysed using the LC-MS/MS method described in Chapter Three, section 3.3.3. Briefly, 25 µL of plasma was added to 500 µL of extraction solvent which consisted of 90% ACN and 10% MeOH containing 2 µM each of the internal standards (D_9-glycine betaine, D_9-trimethylamine-*N*-oxide, D_4-homarine, D_6-DMSP and $^{13}C_2$-arsenobetaine). Samples were vortexed for 5 minutes and centrifuged at 13,000 rpm for 5 minutes, then transferred to 96-well polypropylene microtitre plates and covered until analysis. Plates were stored at -20 ºC and run within 12 hours.

Aqueous solutions of biomarkers were prepared to serve as calibration standards at concentrations that covered the range expected in seal plasma for each analyte: for TMAO and GB 1, 5, 10, 20, 40 and 80 µmol L^{-1}, AsB at concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 1, 5 µmol L^{-1} and homarine and DMSP at concentrations of 0.1, 0.25, 0.5, 1, 5 and 10 µmol L^{-1}. These standards were prepared by independent dilutions of a 10 mM aqueous stock standard for each analyte, split into aliquots, and stored at -80 °C in 1.5 mL microcentrifuge tubes until needed. Then 25 µL of standard was added to 500 µL extraction solvent and prepared as above to run on the LC-MS/MS. The ratio of analyte (TMAO, GB, AsB, DMSP and homarine) peak area divided by the peak area of the corresponding stable isotope-labelled internal standard was calculated and plotted against analyte concentration to form the calibration curve.

5.2.3 Calculation of biomarker fasting limits

TDR data confirmed that no seals entered the water during the first week post partum and therefore did not consume any food during this initial period. Mean plasma concentrations of TMAO, GB, and AsB measured at 7 dpp were used to calculate fasting limits using 1-tailed 95% upper confidence limits from the t-distribution. Biomarker concentrations above these fasting limits were considered to represent feeding. No fasting limit was set for homarine because homarine was not detected in any plasma samples at 2, 3, 6 or 7 dpp. Detection of homarine at subsequent sampling periods above the LOD_s (1.4 µmol L^{-1}) was considered to be an indication of feeding.

5.2.4 TDR analysis

Dive data were downloaded from each TDR in the form of a Wildlife Computers file (.wch) for each female. The software HexDecode.2.02.0017 (Wildlife Computers) was then used to convert the files into comma-delimited text files (.csv). CSV files were imported into excel (version 2007). GMT times recorded by the TDRs was transformed to local McMurdo times (NZ local daylight savings = GMT + 13 hours) and the files were formatted to the appropriate sequence of columns (McMurdo date/McMurdo time/depth). All further analyses and graphical representations were conducted using SigmaPlot (version 11.0). The depth threshold to qualify as a dive was set to five meters. Previous studies on diving of Weddell seals have used a threshold of twelve meters (Burns et al. 1999). For analyses, any dive for which the maximal depth was less than 5 meters was considered to be associated with resting at the surface.

Dive activity in the period (48 hours) immediately prior to blood sampling was compared with biomarker concentrations to examine whether the depth and duration of dives on feeding days (elevated biomarker concentrations at time of sampling) would differ from non-feeding days (no elevated biomarker concentrations at time of sampling). "Feeding day dives" were categorised as dives occurring two days prior and on the day of a blood sample that had elevated concentrations of biomarkers. All other dives were categorised as "non-feeding day dives" but were restricted to the period after seals started actively diving. These analyses were restricted to females that fed during lactation and for which TDR data was available, as not all TDRs were recovered and hence no data exist for these individuals. A Kruskal-Wallis one way ANOVA on ranks using the statistical program SigmaPlot was used to test for differences between the two types of dives.

5.3 Results

5.3.1 Fasting levels

Plasma concentrations were monitored in eight lactating females from 2–3 until 38 ± 1 dpp; 2 females from 2–31 dpp and 2 females from 2 to 24–25 dpp. One female (1043) was sampled only three times, twice during EL and once in LL; no samples could be collected during ML. Calculated fasting limits were 23.7 µmol L^{-1} for TMAO, 0.07 µmol L^{-1} for AsB and 15.1 µmol L^{-1} for GB (Figure 5.1). Females with concentrations above these fasting limits

were considered to be feeding. DMSP was not detected in any females regardless of lactation period. GB levels remained steady throughout lactation and were not significantly different between the three lactation stages (SigmaPlot, repeated measures ANOVA, $F = 1.397$, $P = 0.256$; Table 5.2). Therefore, GB was not used to distinguish between feeding *versus* fasting. Mean plasma TMAO increased throughout lactation and was six times higher in LL compared to EL (Table 5.2). TMAO concentrations were significantly different between the three lactation stages (repeated measures ANOVA, $F = 7.604$, $P = 0.001$). *Ex post facto* comparisons showed a significant difference in TMAO concentrations between LL and EL (Holm-Sidak test, $P = 0.001$, Table 5.2). AsB also differed between the lactation stages (repeated measures ANOVA, $F = 12.506$, $P < 0.001$), with a significant difference between EL and LL (Holm-Sidak test, $P < 0.001$) and ML and LL (Holm-Sidak test, $P = 0.006$). Homarine was not detected in EL (Table 5.2).

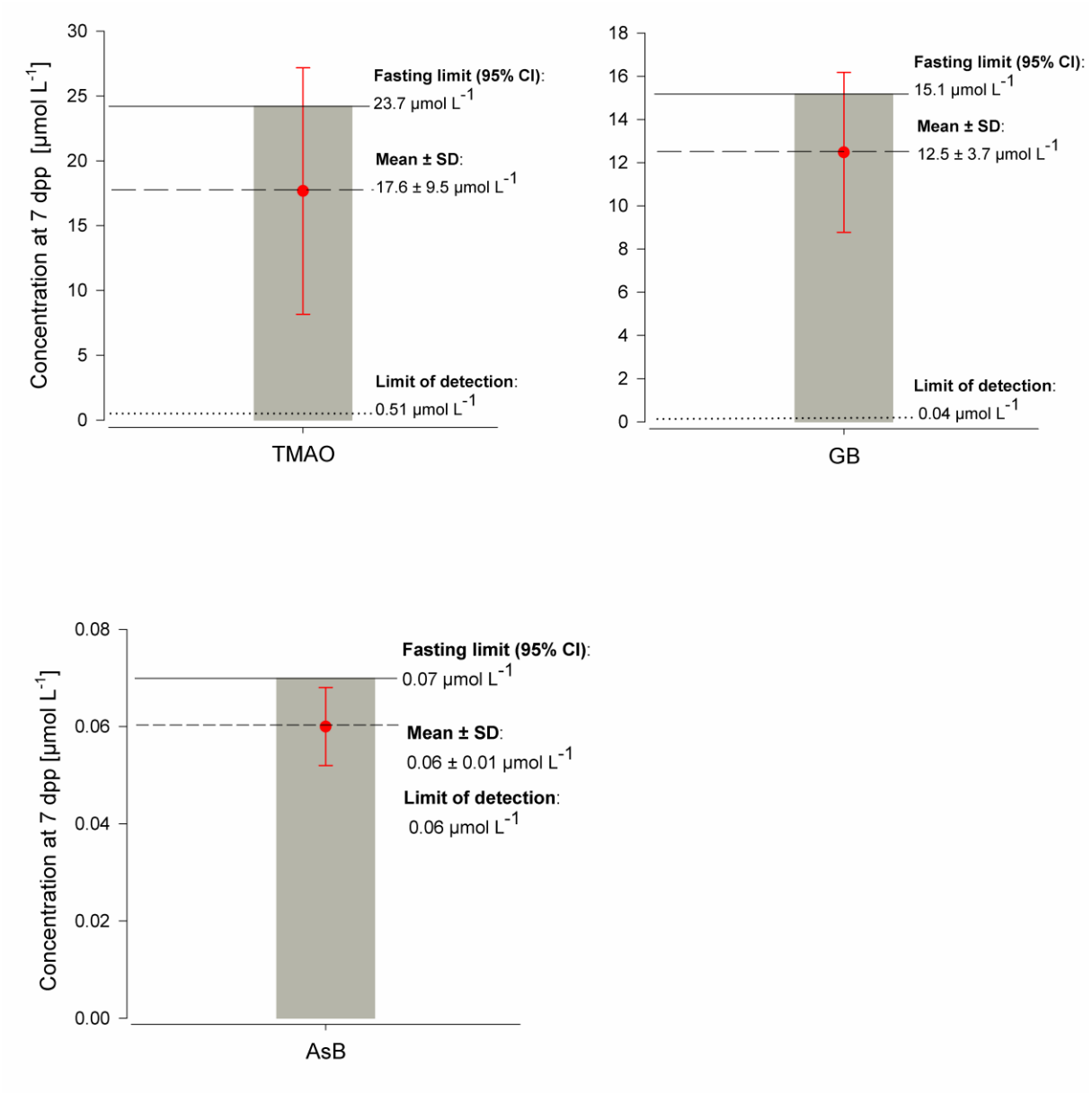


Figure 5.1. Calculated fasting limits for TMAO, GB and AsB at 7 days post partum (dpp). Homarine and DMSP were not detected at 7 dpp.

Table 5.2. Plasma biomarker concentrations (mean ± SD) in female Weddell seals during lactation. Numbers in square brackets indicate median plasma concentrations.

	Early lactation (0-13 dpp)	Mid lactation (14-26 dpp)	Late lactation (≥ 27 dpp)
Number of seals studied	12	11	10
Plasma biomarker concentrations (μmol L^{-1})			
TMAO	39 ± 61 [21]	91 ± 135 [18]	378 ± 378 [47]
AsB	0.17 ± 0.1 [0.15]	0.28 ± 0.3 [0.17]	0.70 ± 0.4 [0.65]
Homarine	Not detected	4.83 ± 6.4 [1.20]	3.13 ± 2.3 [2.15]
GB	16.6 ± 4.2 [16.5]	16.2 ± 5.7 [16.4]	19.6 ± 6.9 [19.0]
DMSP	Not detected	Not detected	Not detected

5.3.2 Plasma biomarker concentrations and dive activity

Diving activity and plasma biomarker concentrations were monitored concurrently in ten lactating females (Table 5.3); TDRs were not recovered for females 5981 and 622 as these seals left the area before the experiment was concluded. In total, 6,039 dives were recorded for the ten females for which TDRs were recovered. Table 5.4 presents the mean and maximum values of dive parameters for each female. Females dived on average to 56 m, but large individual variations were observed (range 11–128 m, Table 5.4). In all females, more than 50% of dives occurred in depths < 50 meters (Figure 5.2). The mean duration of dives also varied amongst individuals with an overall average of 5.2 minutes (range 2.0–11.1 minutes, Table 5.4).

Elevated levels of TMAO, AsB and homarine indicated that nine out of twelve females fed during lactation (females 40, 105, 115, 265, 622, 819, 946, 5891, 1043), while for three females evidence for feeding was inconclusive (females 479, 515, 5746). In females that fed during lactation, two females starting feeding between 7–12 dpp (females 946 and 5891), four females between 12–19 dpp (females 40, 115, 265 and 622), one female between 19–24 dpp (819) and two females between 19–31dpp (105 and 1043). Females that fed during lactation dived more frequently to deeper depths than those did not feed during lactation (Figure 5.2).

Table 5.3. Onset of diving in female Weddell seals compared to elevated plasma biomarker concentrations during lactation. Numbers in brackets indicate the day post partum (dpp) that biomarker concentrations were above the fasting limit.

Seal ID	Diving > 5m (dpp)	Diving > 50m (dpp)	TMAO µmol L^{-1} (dpp)	AsB µmol L^{-1} (dpp)	Homarine µmol L^{-1} (dpp)
40	12	17	204 (19)	0.16 (19)	1.2 (19)
105	9	26	47 (38)	no increase	no increase
115	11	11	467 (18)	0.24 (18)	no increase
265	10	10	374 (19)	0.34 (19)	no increase
479	14	32	no increase	no increase	no increase
515	14	31	no increase	no increase	no increase
622	TDR not recovered		27 (12)	0.35 (17)	no increase
819	13	13	19 (24)	0.12 (24)	1.10 (24)
946	9	9	163 (12)	0.08 (12)	2.05 (31)
1043	17	17	98 (31)	0.06 (31)	0.15 (31)
5746	17	17	33 (9)[a]	no increase	no increase
5891	TDR not recovered		349 (12)	0.22 (12)	no increase

[a]TMAO concentrations were above the fasting limit at 9 dpp but the seal did not enter the water until 17 dpp

Table 5.4. Dive parameters of female Weddell seals during the lactation period, October to December 2006. Means are ± standard deviations.

Seal ID	# of dives	Mean depth (m)	Max depth (m)	Mean duration (min)	Max duration (min)
40	820	80 ± 115	399	7.2 ± 9.0	29.1
105	477	30 ± 65	369	3.3 ± 4.2	25.5
115	1142	35 ± 69	382	3.4 ± 4.7	34.1
265	410	124 ± 153	423	9.4 ± 10.3	32.0
479[a]	310	8.9 ± 13.6	183	1.3 ± 1.5	26.4
515	256	11 ± 17	176	2.1 ± 1.3	10.4
819	1133	67 ± 90	389	6.2 ± 6.1	27.4
946	855	128 ± 137	419	11.1 ± 10.5	33.3
1043	247	41 ± 89	384	4.2 ± 9.5	41.0
5746	293	35 ± 79	364	3.3 ± 5.0	24.3
Total	6039				
Overall mean		56 ± 42.9		5.2 ± 3.2	

[a] Dive parameters for seal 479 from 2-38 dpp.

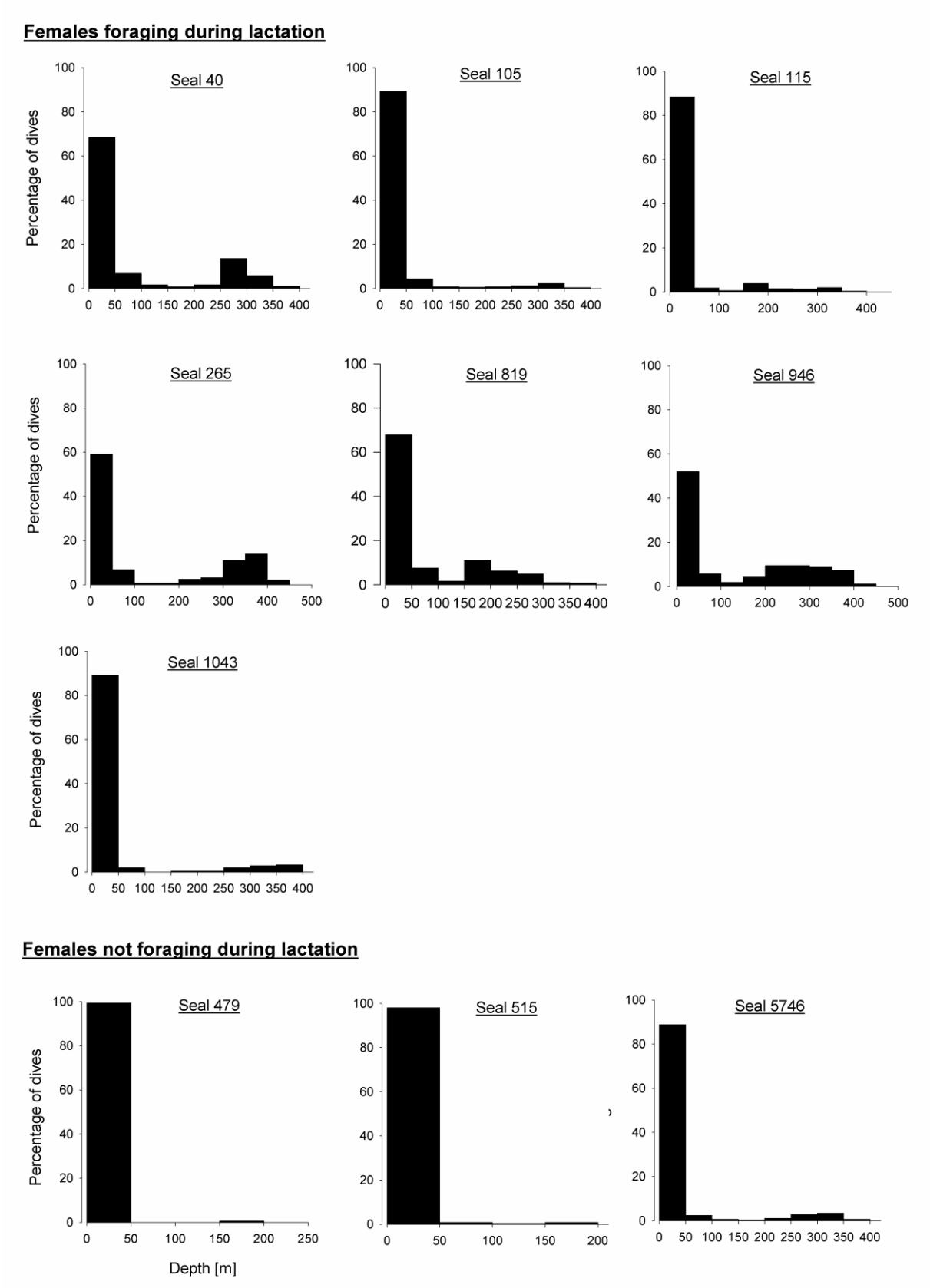


Figure 5.2. Distribution of dives by depth (in meters) for female Weddell seals studied in 2006. Dive depths for seal 479 from 2-38 dpp.

5.3.3 Females foraging during early to mid-lactation

Seven females started feeding during early to mid-lactation, of which TDR data was available for five females. Females 946 and 5891 both had plasma biomarker concentrations in excess of the fasting limits at 12 dpp (female 946, 202 µmol L^{-1} TMAO, 0.15 µmol L^{-1} AsB and female 5891, 277 µmol L^{-1} TMAO and 0.22 µmol L^{-1} AsB). Both females also had the highest initial post partum mass (Table 5.1). Female 946 made dives > 50 m (three dives were > 200 m) as soon as 9 dpp and made consistently deep dives > 300 m until the end of the study period (39 dpp, Figure 5.3 and 5.4). TMAO plasma concentrations were highest at 31 dpp at 924 µmol L^{-1}. Both homarine and AsB plasma were elevated at the end of lactation (Figure 5.3). Median dive depth for seal 946 was 41 m and maximum depth recorded was 419 m (Table 5.4). TDR data was not recovered for female 5891. Both plasma TMAO and AsB concentrations were elevated between 12 and 18 ddp in female 5891 but then fell below the feeding limits for the rest of the study period (Figure C1.1, Appendix C). Homarine was not detected at any bleed.

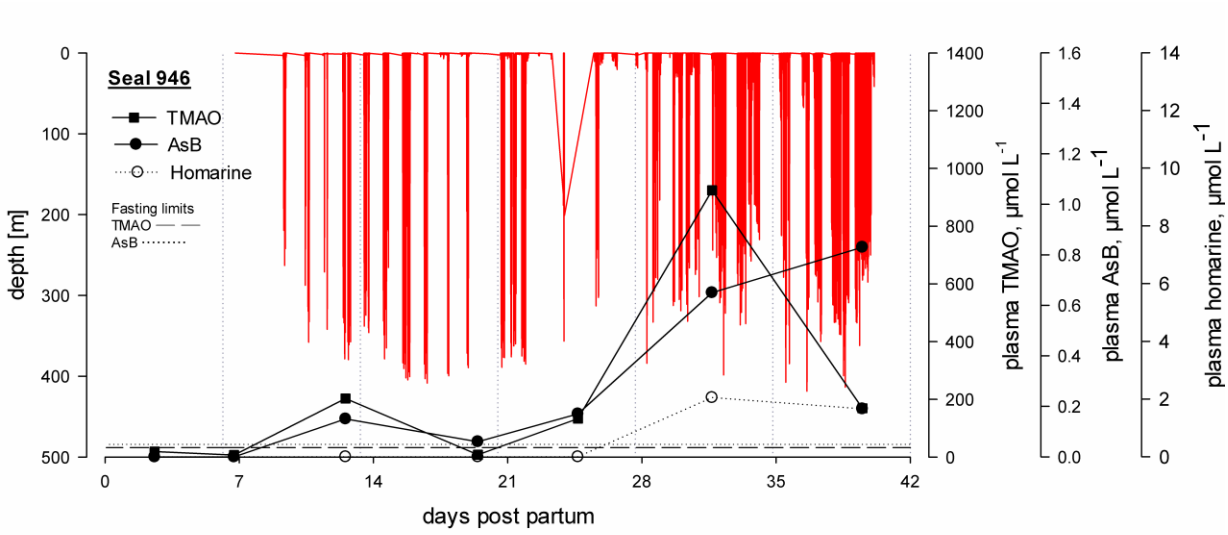


Figure 5.3. Comparison of dive activity with plasma biomarker concentrations in female 946 during the lactation period.

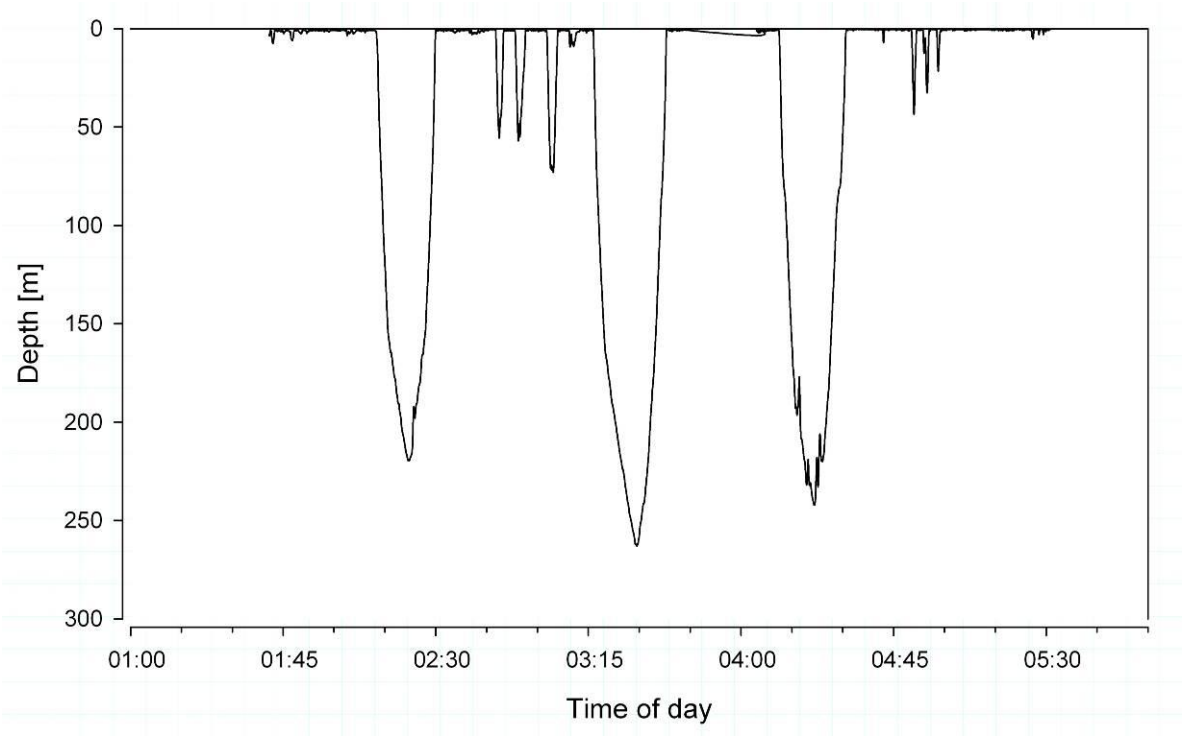


Figure 5.4. Dive profile of female 946 on 2 November 2006 at 9 dpp (first day that the seal entered the water).

Female 946 made significantly deeper dives on feeding days compared to non-feeding days (Figure 5.5, Table 5.5). The numbers of dives between 200-350 m increased, which suggests that is where she was feeding. There was no difference in dive duration between feeding and non-feeding day dives (Table 5.5).

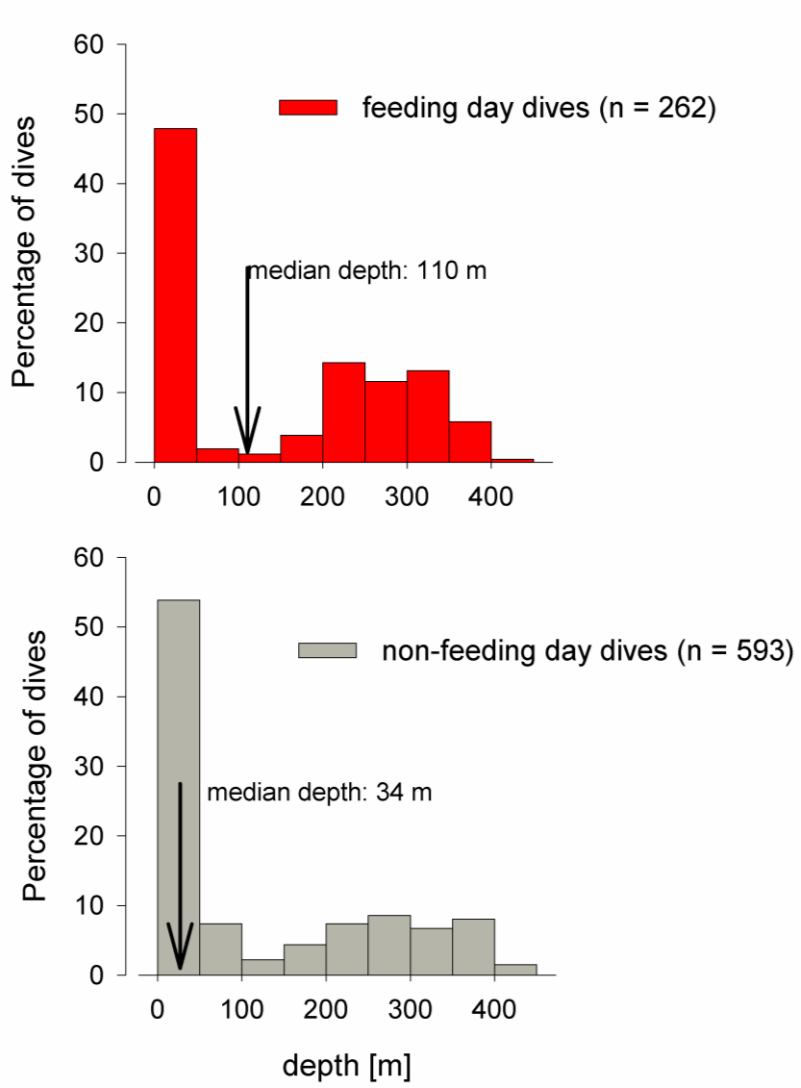


Figure 5.5. Distribution of feeding day and non-feeding day dives for female 946 over the lactation period.

Table 5.5. Comparison of depth and duration between non-feeding and feeding day dives in females that fed during the lactation period and for which TDR data was available.

	Depth [m]							Duration [min:sec]						
	Non-feeding day dives			Feeding day dives				Non-feeding day dives			Feeding day dives			
Seal ID	**Mean**	**Max**	**Min**	**Mean**	**Max**	**Min**	**Sig.**	**Mean**	**Max**	**Min**	**Mean**	**Max**	**Min**	**Sig.**
40	73 ± 109	376	5	94 ± 124	399	5	p = 0.078	6:48 ± 8:42	29:00	0:20	8:18 ± 9:28	29:10	0:20	p = 0.067
105	28 ± 65	369	5	38 ± 67	356	5	*p < 0.001*	3:18 ± 4:19	25:50	0:20	4:10 ± 4:03	22:20	0:40	*p < 0.001*
115	33 ± 66	382	5	60 ± 99	344	5	p = 0.963	3:33 ± 4:49	34:10	0:20	5:30 ± 5:56	23:20	0:40	*p < 0.001*
265	118 ± 149	423	5	145 ± 167	423	5	*p = 0.021*	9:17 ± 10:17	32:00	0:20	11:05 ± 11:19	32:00	0:40	p = 0.221
819	57 ± 80	346	5	93 ± 1096	389	5	*p < 0.001*	5:48 ± 5:51	24:00	0:20	7:49 ± 6:59	27:40	0:20	*p < 0.001*
946	121 ± 138	419	5	145 ± 137	411	5	*p = 0.028*	10:40 ± 10:57	33:30	0:20	12:31 ± 10:38	33:00	0:30	p = 0.294
1043	40 ± 90	384	5	43 ± 87	369	5	*p < 0.001*	4:28 ± 10:04	22:50	0:20	3:49 ± 5:45	25:20	0:30	*p = 0.028*

Female 40 made few shallow dives (< 50 m) from 12–16 dpp and afterwards consistently made dives > 50 m from 17–38 dpp (Figure 5.6). Plasma biomarker concentrations remained below the fasting limits during EL. Elevated biomarker levels appear to be concurrent with increased dive activity and diving to deeper depths (Figure 5.7). Plasma concentrations of TMAO, AsB and homarine were above fasting limits at 19 dpp (Table 5.3). Biomarker levels remained above fasting limits throughout ML and LL. Female 40 also had the highest concentration of plasma homarine at 12.2 µmol L^{-1} (26 dpp, Figure 5.6). Overall median dive depth was 12 m and maximum depth measured was 399 m (Table 5.4).

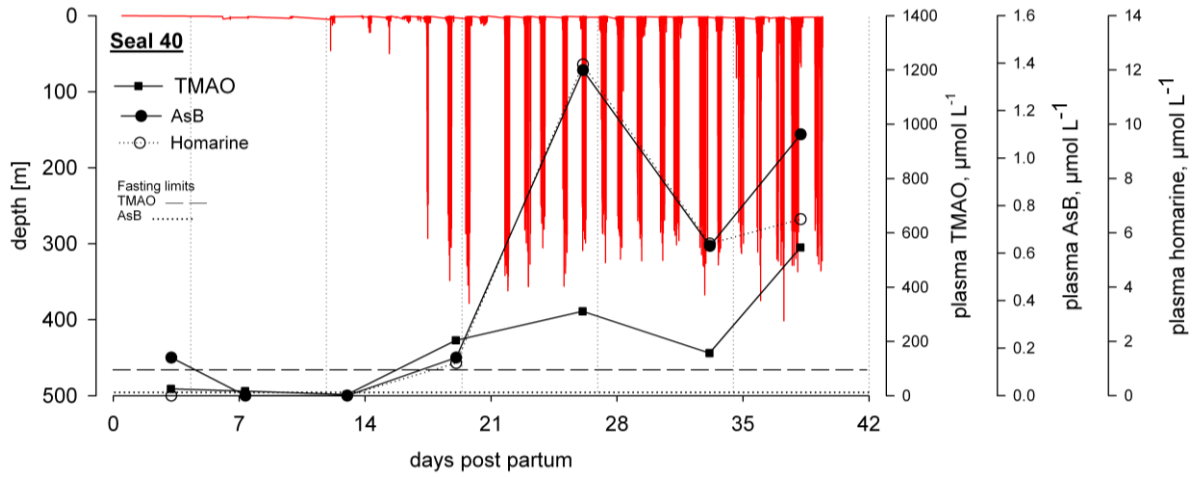


Figure 5.6. Comparison of dive activity with plasma biomarker concentrations in female 40 over the lactation period.

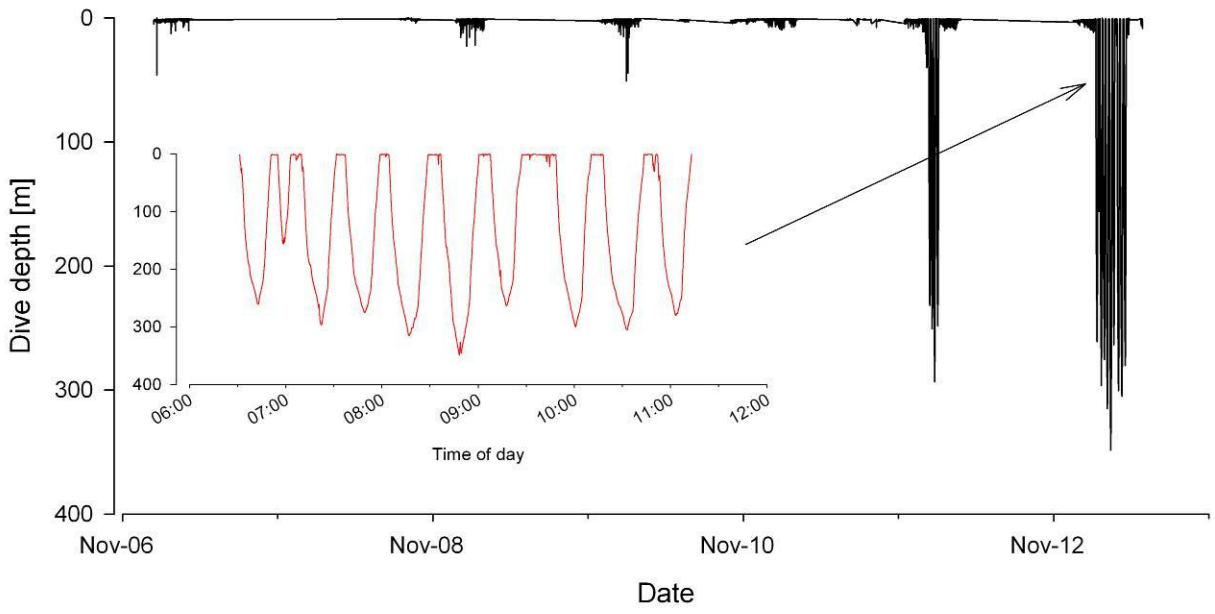


Figure 5.7. Dive profile of female 40 between 6-12 November (13 to 19 dpp). Seal 40 made several deep dives (inset) on 12 November (19 dpp) between 0600 and 1200 hours. The total dive bout lasted for almost 4.5 hours. A blood sample taken on 12 November (19 dpp) showed plasma TMAO, homarine and AsB levels were above the fasting limit (Figure 5.6).

An increase in the number of dives between 350 and 400 m was observed in feeding day dives for female 40, suggesting that she was feeding at these depths (Figure 5.8). However, there were no significant differences in the depth or duration between feeding day dives and non-feeding day dives (Table 5.5).

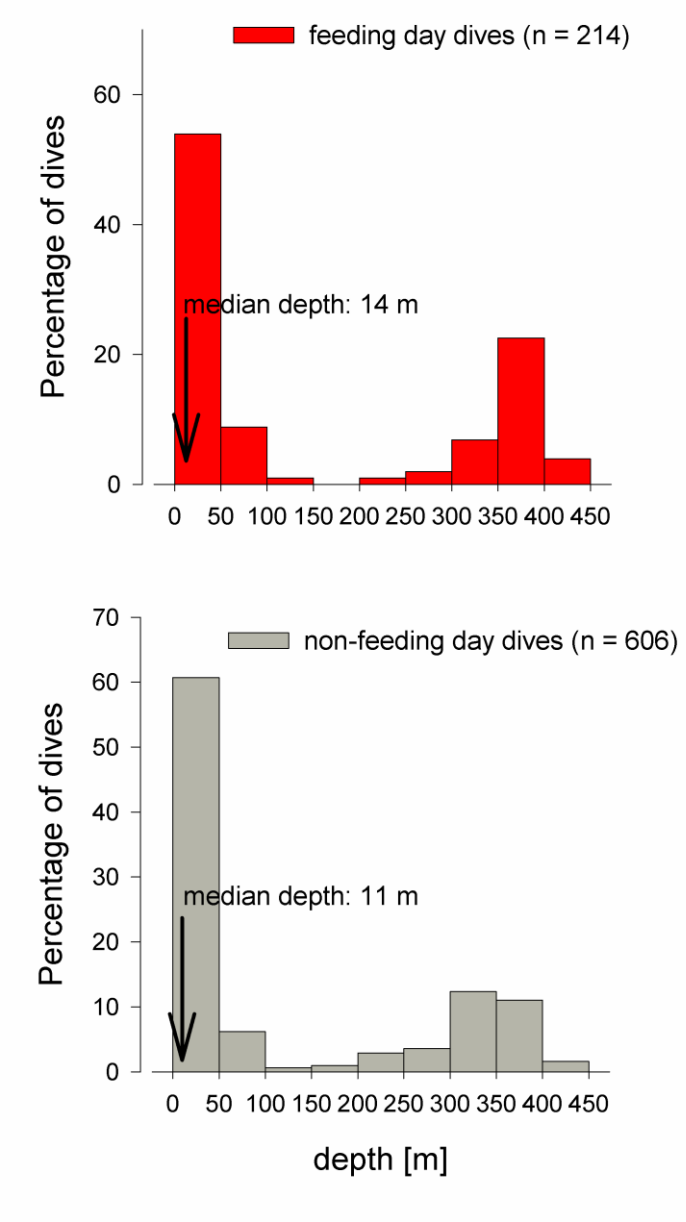


Figure 5.8. Distribution of feeding day and non-feeding day dives for female 40 over the lactation period.

Female 115 entered the water at 11 dpp and made several dives > 5 m, with two dives > 150 m (Figure 5.9). Afterwards, seal 115 did not enter the water until 4 days later at 15 dpp. Plasma concentrations of TMAO were elevated at 2 dpp (61 µmol L^{-1}) but remained below the fasting limits in EL (Figure 5.9). Plasma AsB and TMAO was elevated in ML at 18 dpp (0.24 and 467 µmol L^{-1}, respectively) and then declined below the fasting limits until the end of the study (39 dpp) even though the seal continued to dive regularly and at deep depths up to 32 dpp (Figure 5.9). Afterwards, dives were considerably shallower (< 50 m). Maximum

dive depth was 382 m and median dive depth was 12 m (Table 5.4). AsB was only above the fasting limit at 18 dpp (0.24 µmol L^{-1}) and homarine was not detected at any bleed.

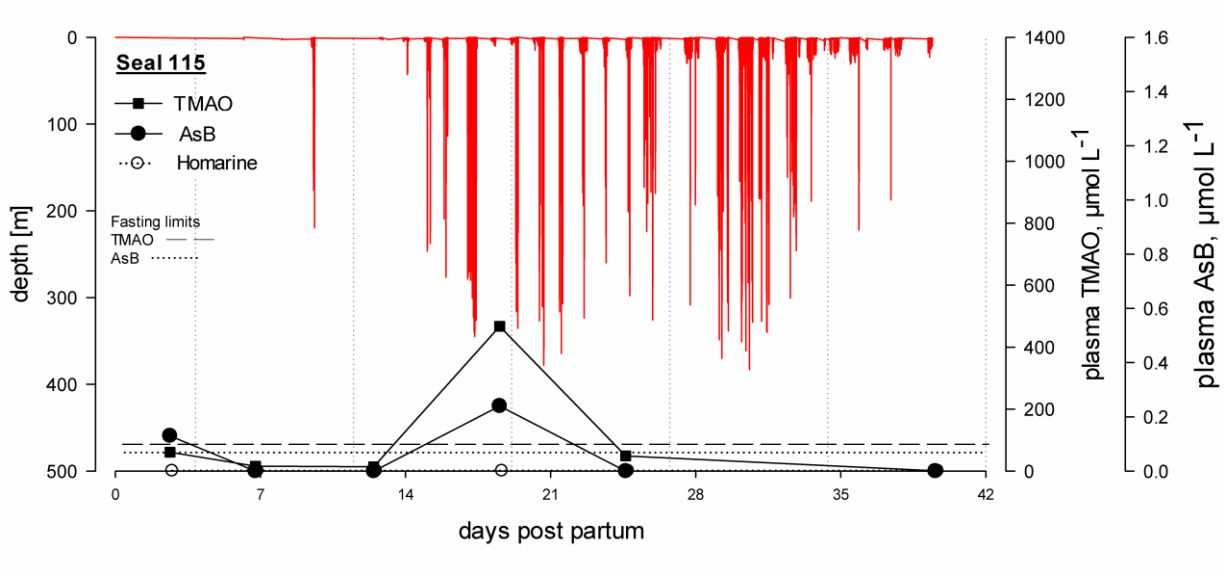


Figure 5.9. Comparison of dive activity with plasma biomarker concentrations in female 115 over the lactation period.

Although the number of dives to 200 and 350 m increased during feeding day dives (Figure 5.10), there were no significant differences in dive depth between feeding and non-feeding day dives for female 115 (Table 5.5). However, female 115 did dive longer during feeding day dives compared to non-feeding day dives (Table 5.5).

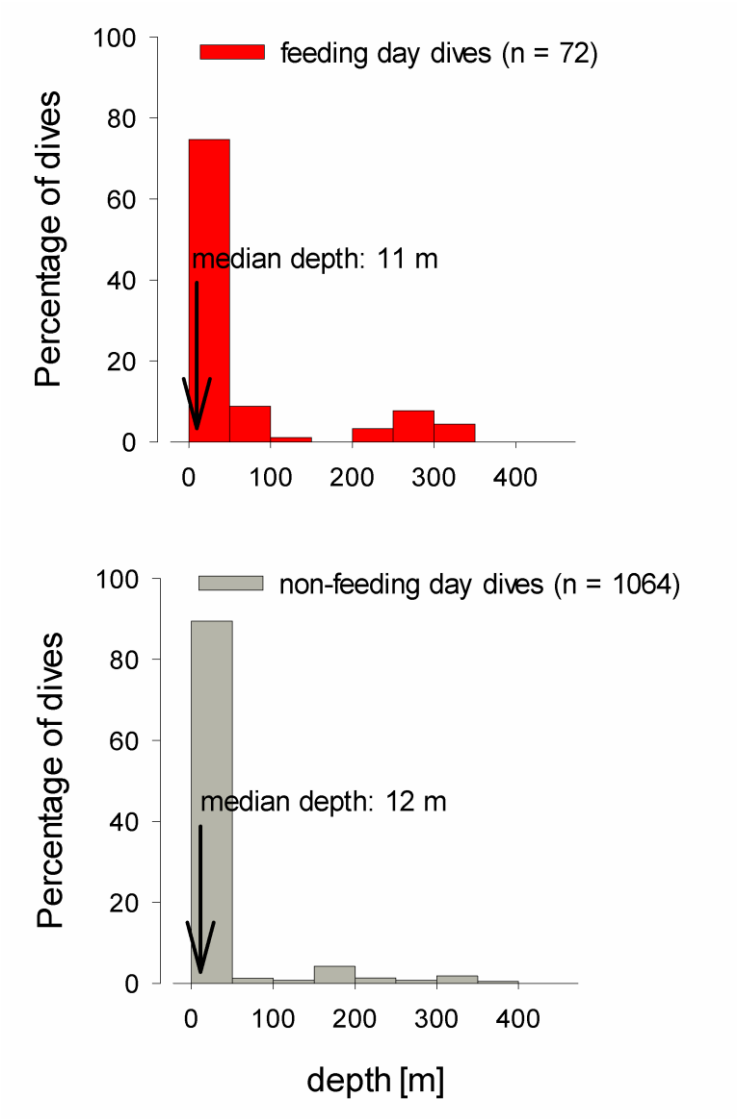


Figure 5.10. Distribution of feeding day and non-feeding day dives for female 115 over the lactation period.

Female 265 entered the water at 10 dpp and made five dives, three of which were to depths > 200 m (Figure 5.11). Afterwards female 265 did not start diving consistently until 14 dpp and on most days dives exceeded > 50 m until the end of the study period. Plasma biomarker concentrations varied over the lactation period with highest concentrations at 19 and 38 dpp (Figure 5.11). TMAO and AsB were elevated at 19 dpp but not homarine. Homarine was not detected until 38 dpp. Overall median dive depth was 16 m and maximum dive depth measured was 423 m (Table 5.4).

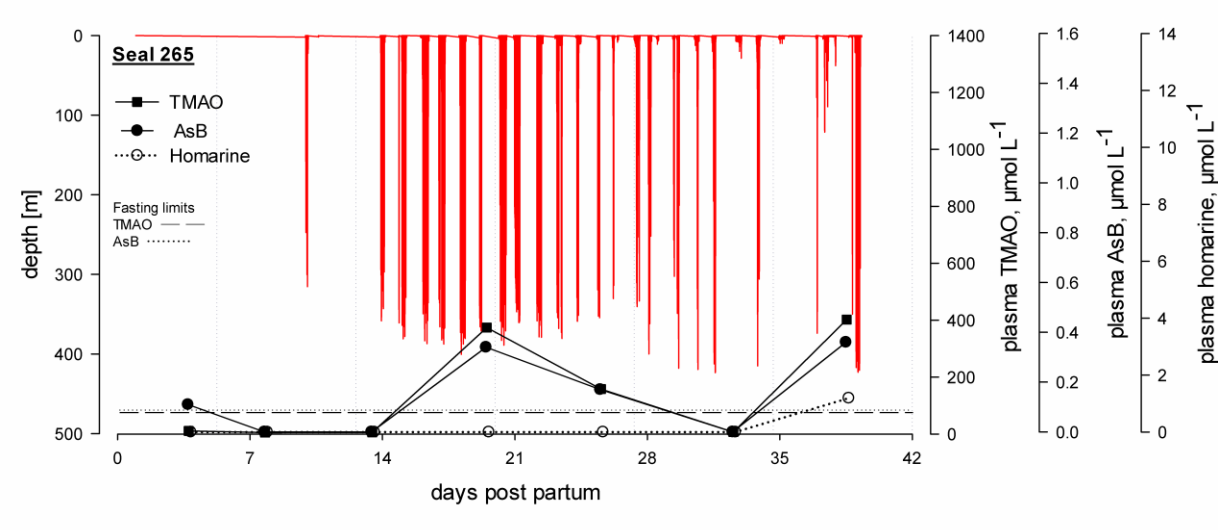


Figure 5.11. Comparison of dive activity with plasma biomarker concentrations in female 265 over the lactation period.

There were significant differences in dive depth between feeding day and non-feeding day dives (Table 5.5). Female 265 made more dives between 350-400 m on feeding days suggesting she was feeding at this depth (Figure 5.12). There were no significant differences in the duration of dives between feeding and non-feeding day dives (Table 5.5).

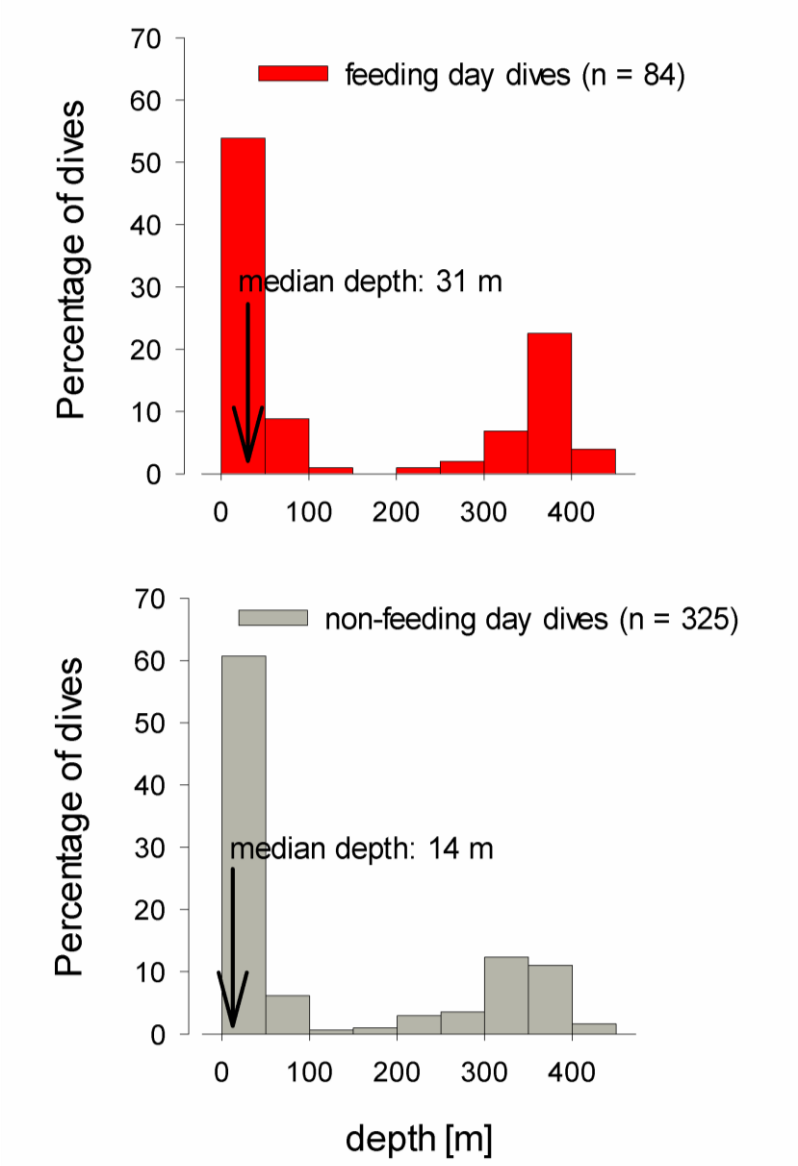


Figure 5.12. Distribution of feeding day and non-feeding day dives for female 265 over the lactation period.

Female 819 made multiple dives at 13 dpp (> 100 m) and afterwards less frequent and shallow dives until 23 dpp. Dives exceeded 50 m on most days until the end of the study period. The increase in dive frequency at 23 dpp was concurrent with the increase in plasma biomarker concentrations (Figure 5.13). Plasma TMAO, AsB and homarine levels were above the fasting limits at 24 dpp (Figure 5.13). Plasma concentrations remained elevated in LL with concentrations of plasma TMAO exceeding 1,000 µmol L^{-1}. Overall median dive depth was 13 m and maximum dive depth measured was 390 m (Table 5.4).

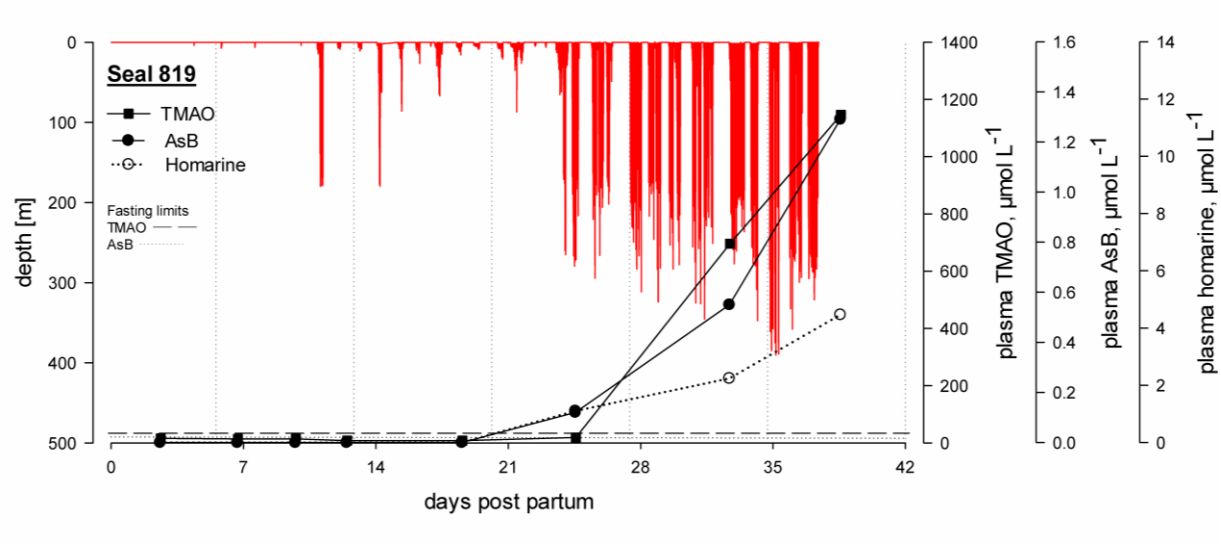


Figure 5.13. Comparison of dive activity with plasma biomarker concentrations in female 819 over the lactation period.

There was a significant difference in both the depth and duration between feeding and non-feeding day dives for female 819 (Table 5.5). The number of dives to depths between 150 and 300 m increased during feeding days (Figure 5.14) while the duration of dives increased from 5:48 to 7:49 minutes (Table 5.5).

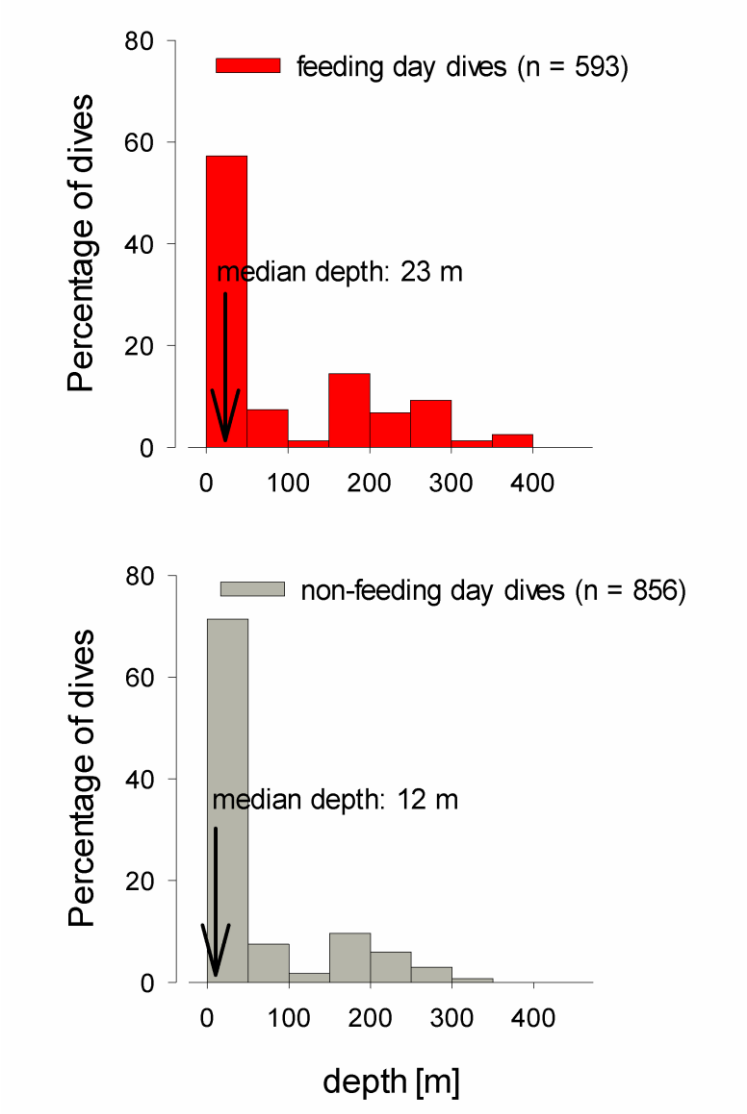


Figure 5.14. Distribution of feeding day and non-feeding day dives for female 819 over the lactation period.

TDR data was not recovered for female 622 and plasma biomarker concentrations were only monitored for a shorter period (2 to 25 dpp). Plasma TMAO rose above the fasting limit at 12 dpp (28 µmol L^{-1}) and peaked at 17 dpp (370 µmol L^{-1}). Plasma AsB was also above the fasting limit at 17 dpp (0.35 µmol L^{-1}). Both AsB and TMAO were below the fasting limit at the last blood sample (25 dpp). Homarine was not detected at any bleed (Figure C1.2, Appendix C).

5.3.4 Females foraging during late-lactation

Two females started feeding at the very end of lactation (105 and 1043). Three females may or may not have started to feed at the very end of lactation because blood samples were only taken until 37-39 days post partum, and females 479, 515 or 5746 may have started feeding thereafter, but before weaning their pups.

Only TMAO plasma concentrations were above the fasting limit in LL in female 105 (Figure 5.15). Plasma TMAO concentrations were above the fasting limit at 2 dpp (Figure 5.15) but remained below the fasting limit until 38 dpp when the concentration (47 µmol L^{-1}) exceeded the fasting limit. However, TMAO concentrations at 25 and 31 dpp were 18 and 19 µmol L^{-1}, respectively, a three-fold increase of the plasma concentration (5 µmol L^{-1}) measured at 18 dpp, which suggests this female may have started feeding between 18 and 25 dpp, even though these concentrations were below the calculated fasting limit (Figure 5.15, inset). Plasma concentrations of AsB were above the fasting limit at 2 dpp (0.22 µmol L^{-1}) but fell below the LOD until the end of the study. Homarine was not detected at any bleed. Female 105 made a small number of shallow dives as early as 9 dpp (Figure 5.15). Dives exceeded > 50 m and became more frequent from 30 dpp onwards until the end of the study period which was concurrent with the increase in TMAO concentrations. Overall median dive depth was 9 m and maximum dive depth measured was 369 m (Table 5.4).

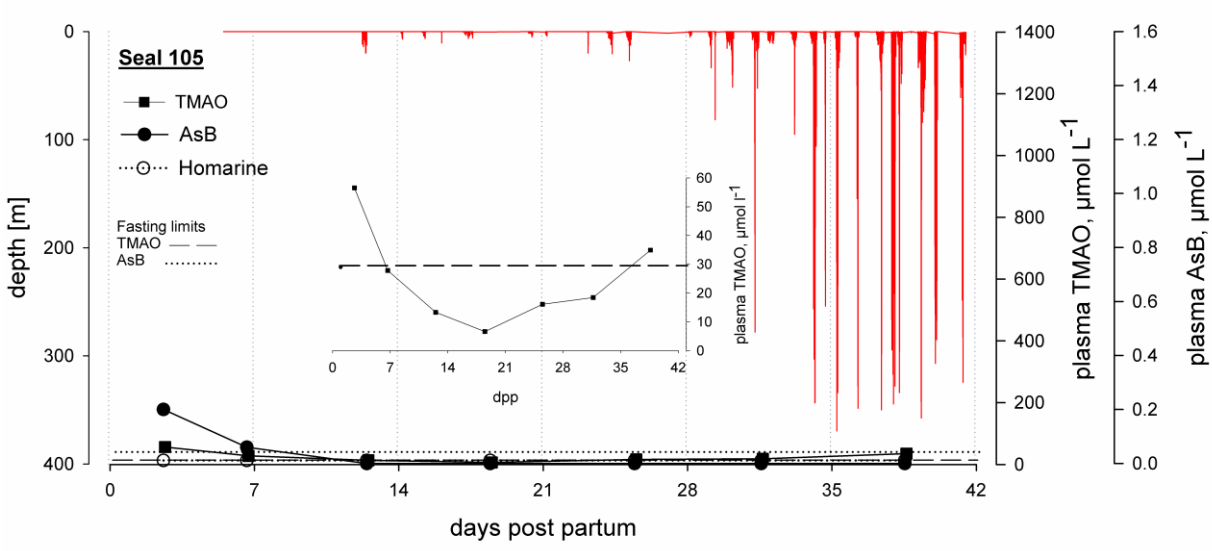


Figure 5.15. Comparison of dive activity with plasma biomarker concentrations in female 105 over the lactation period. Plasma TMAO (inset).

There was a significant difference in both the depth and duration between feeding and non-feeding day dives for female 105, even though only 69 dives were considered feeding dives compared to 408 non-feeding dives (Table 5.5, Figure 5.16). Female 105 started diving to depths between 150 and 250 m during feeding dives, suggesting she may have been feeding at these depths (Figure 5.16).

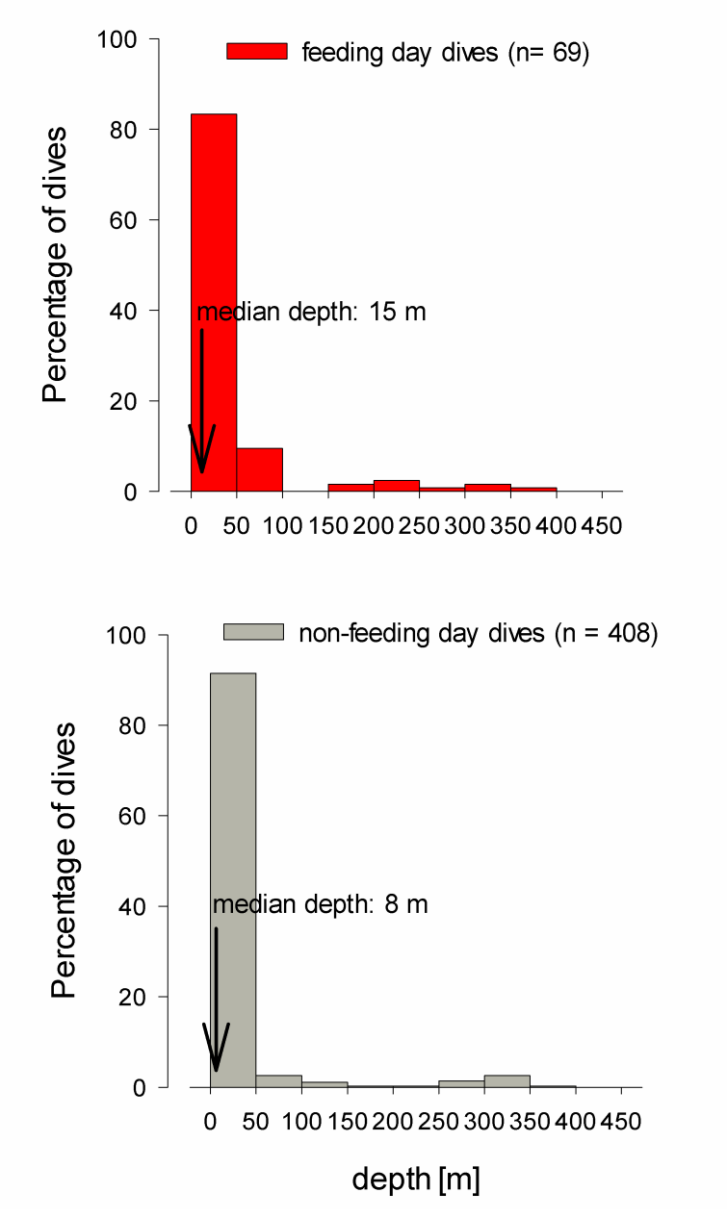


Figure 5.16. Distribution of feeding day and non-feeding day dives for female 105 over the lactation period.

Female 1043 was difficult to work on, so she was only sampled for plasma biomarkers twice during EL (2 and 6 dpp) and once during LL (31 dpp). TMAO, AsB and homarine concentrations were above the fasting limit at 31 dpp. However, it is possible that this female started feeding during ML based on dive activity (Figure 5.17). Female 1043 did not start diving until ML at 17 dpp when dives exceeded 5 and 50 m (Table 5.3). Overall median dive depth was 10 m and maximum dive depth measured was 384 m (Table 5.4).

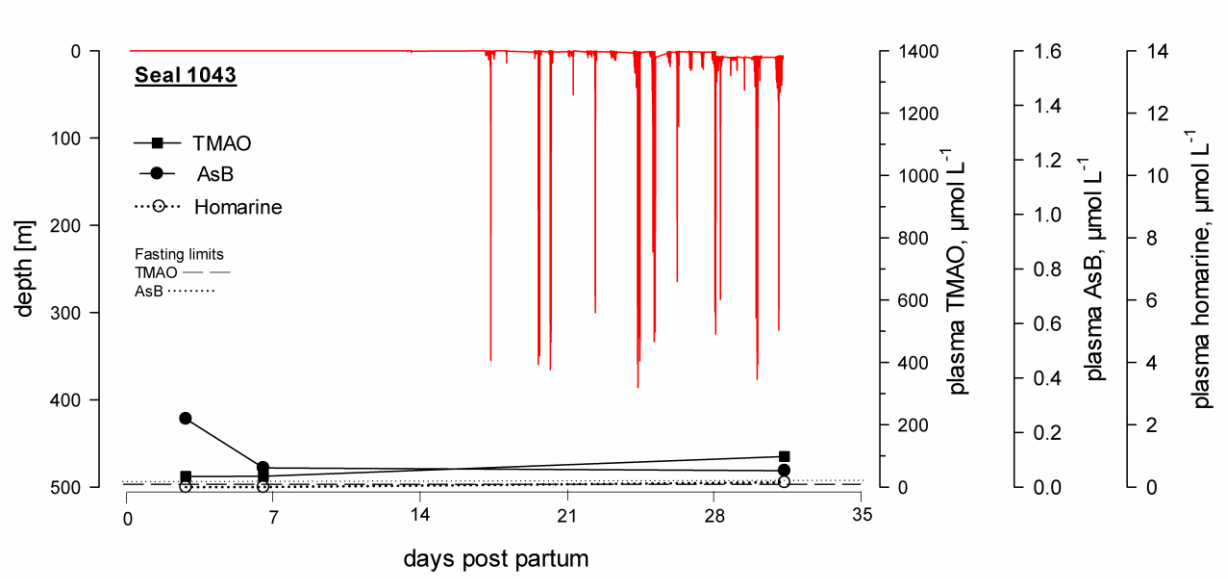


Figure 5.17. Comparison of dive activity with plasma biomarkers concentrations in female 1043 over the lactation period

Plasma AsB concentrations in female 5746 were above the fasting limit at 2 dpp but then fell below the fasting limit or were not detected for the rest of the study period. There was an anomalous increase in plasma TMAO levels at 9 (36 µmol L^{-1}) and 12 dpp (41 µmol L^{-1}) even though female 5746 did not start diving until 17 dpp (Figure 5.18). Female 5746 made deeper dives (> 200 m) on 17, 21, 25, 26, 27, 28, 29, 34, 36 and 37 dpp, but plasma concentrations never rose above the fasting limits in LL (Figure 5.18). Homarine was not detected at any bleed. Overall median dive depth was 8 m and maximum dive depth measured was 364 m (Table 5.4). Female 5746 was the oldest seal in this study (24 years, Table 5.1).

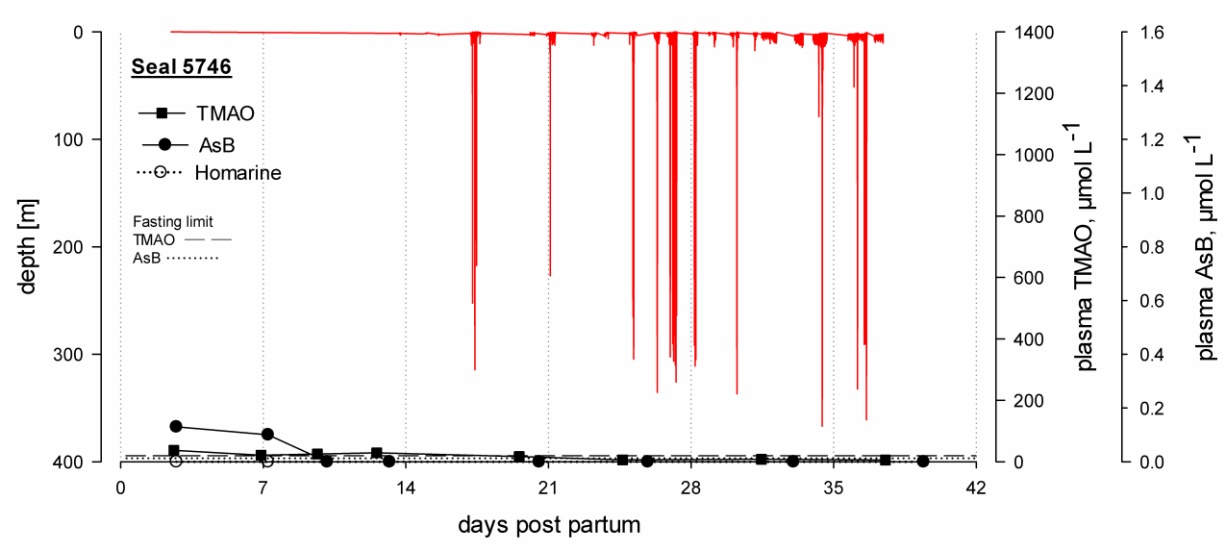


Figure 5.18. Comparison of dive activity with plasma biomarker concentrations in female 5746 over the lactation period.

Plasma AsB concentrations were above the fasting limit at 2 dpp (0.23 µmol L^{-1} AsB) in female 479. Plasma AsB then declined below the fasting limit or was not detected for the remainder of the study. There was an anomalous increase in plasma TMAO levels at 12 dpp (13 µmol L^{-1}) even though this value is below the fasting limit and female 479 did not dive > 5 m until 14 dpp (Table 5.3). Homarine was not detected. Female 479 made several shallow dives < 50 m between 13 and 31 dpp (Figure C1.3, Appendix C). Dives exceeded 100 m depth at 31 and 42 dpp, but unfortunately blood samples were only collected until 24 dpp (this seal was dropped from the experiment because she spent her entire time in a slush pool next to a big crack, precluding capture) so it is not known if female 479 may have started feeding in LL.

Plasma TMAO, AsB and homarine concentrations were all above the fasting limit at 2 dpp in female 515, but declined throughout the study with both AsB and homarine concentrations below the LOD_s. Female 515 made few shallow dives throughout the study period (Figure C1.4, Appendix C). Overall median dive depth was 8 m and maximum dive depth measured was 176 m (Table 5.4).

5.3.5 Method comparison

The results obtained for plasma TMAO and AsB using LC-MS/MS in this study were compared to those obtained previously using HS-GC (TMAO) and GF-AAS (AsB) on the same set of plasma samples (Eisert and Oftedal, unpublished data). There was good agreement between TMAO, although HS-GC recovered more TMAO at the high end of the assay (Passing Bablock agreement test, Figure 5.19a). There was also good agreement between methods for AsB (Passing Bablock agreement test, Figure 5.19b).

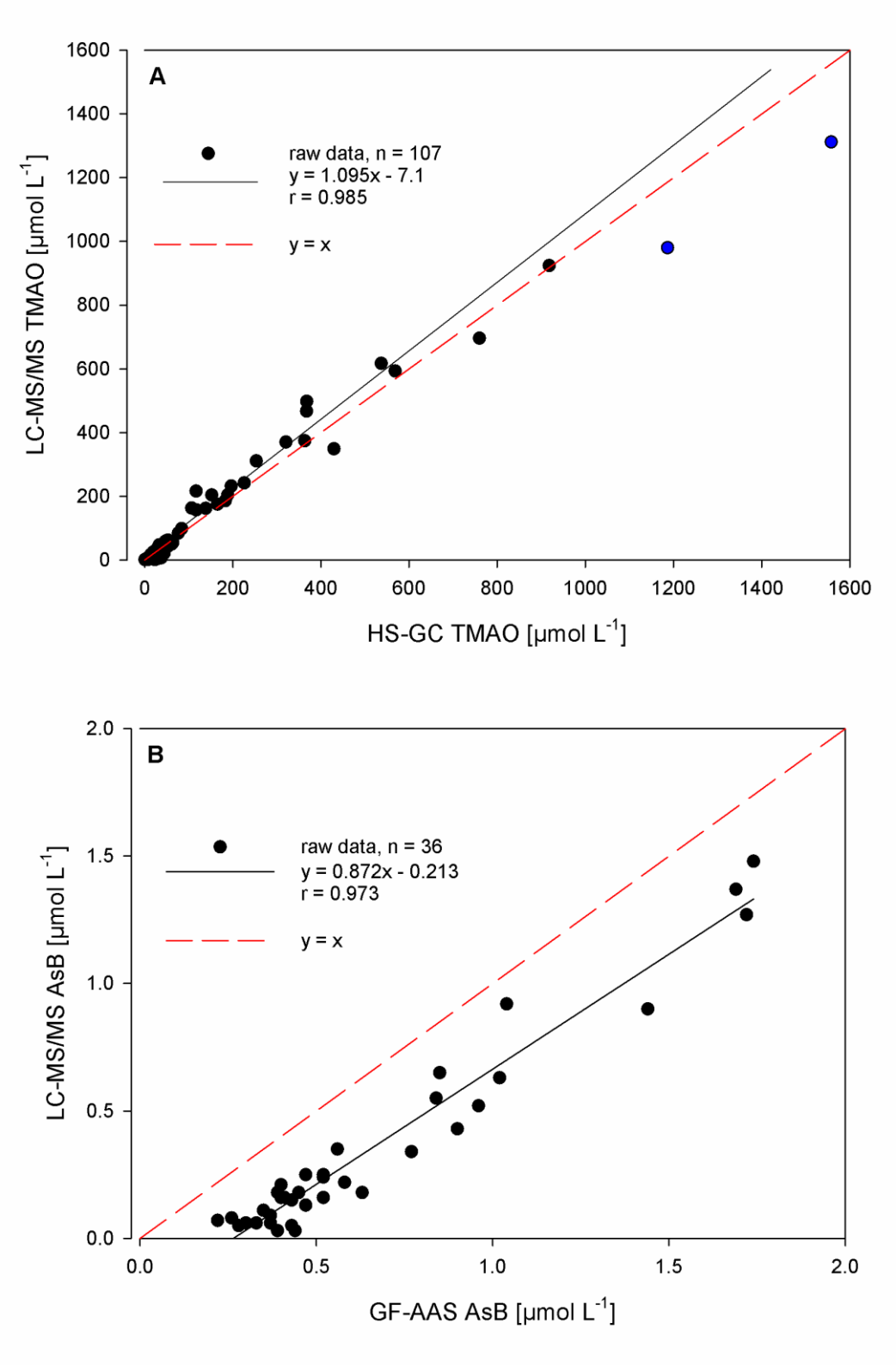


Figure 5.19. Comparison of TMAO in lactating Weddell seals by HS-GC and LC-MS/MS (A) and of AsB by GF-AAS and LC-MS/MS (B).

5.4 Discussion

The work presented here provides a better understanding on the foraging ecology of female Weddell seals during lactation through dietary biomarkers and dive data. The results suggest that feeding during lactation may be more prevalent and initiated at an earlier stage than previously thought. However, the onset of foraging and dive behaviour amongst individuals is highly variable. Two females (946 and 5891) started feeding between 9 and 12 days post partum which is the earliest recorded for lactating Weddell seals, although there was evidence that feeding occurred as early as 9 dpp in Eisert et al. (2005). The rest of the study population started feeding in mid lactation, approximately three weeks post partum, which is consistent with the biomarker results of Eisert et al. (2005) and observed dive activity by Hindell et al. 2002. These results provide more evidence that Weddell seals may indeed follow an income lactation strategy, similar to the harbour seal, where all females start feeding but the onset varies between individuals (Bowen et al. 2001). However, Bowen et al. 2001 found that diving effort was inversely proportional to female postpartum body mass during late lactation with lighter females diving longer and to greater depths compared to heavier females. By comparison, the lightest female in this study (female 1043, 343 kg) made only 45 dives during late lactation to an average depth of 47 meters, while the heaviest and earliest-diving seal in this study (female 946) made 407 dives during late lactation with an average depth of 127 meters. Female 946 also started feeding the earliest which is somewhat surprising given that a capital breeding strategy should favour females with a larger body size (Oftedal 2000). Similar trends have been found by Testa et al. (1989) and Wheatley et al. (2008). Although the present study is based on a few animals and only a single season, there appears to be no simple inverse relationship between female body mass and feeding, suggesting that lactation strategies in Weddell seals is determined by several, complex factors.

The biomarkers measured in this study (except for GB) will only be present if a seal is feeding. In females that fed (females 40, 105, 115, 265, 622, 819, 946, 5891, 1043) the presence of plasma TMAO in all females indicates they were likely consuming fish because TMAO is present in *Pleuragramma antarcticum* (79 mmol kg^{-1}), which is considered their main prey item in McMurdo Sound (Castellini et al. 1992, Burns et al. 1998), as well as in high quantities in the Antarctic toothfish, *Dissostichus mawsoni* (150 mmol kg^{-1}). Unfortunately, at this stage the biomarker method cannot determine what fish species seals may be preying upon solely based on TMAO because TMAO is present in high

concentrations of *c.* 70-160 mmol kg^{-1} in all fish measured from McMurdo Sound (Chapter Four, Table 4.2). Lactating females have been shown to utilise the entire water column (Hindell et al. 2002), foraging at pelagic and benthic habitats as well as at the under ice surface (Davis et al. 1999, Sato et al. 2002, Davis et al. 2004). Therefore, it is possible that females will acquire TMAO by consuming a variety of fish species, although *D. mawsoni* is thought to be depleted near breeding colonies (Testa et al. 1985). Since AsB is virtually ubiquitous in marine organisms, it is also likely to be present in seal blood regardless of what type of prey a seal is eating.

GB plasma levels remained steady throughout lactation and could not be used as a biomarker for detecting feeding *versus* fasting in Weddell seals, even though GB appeared to be a suitable taxon specific biomarker for identifying cephalopods in the diet based on the results in Chapter Four. DMSP was the only compound that was not detected in seal plasma. This is somewhat surprising considering that DMSP was found in concentrations up to 1 mmol kg^{-1} (Chapter Four, Table 4.2) in *Trematomus pennellii*, *T. hansoni* and *T. bernacchii* as well as the cryopelagic fish *P. borchgrevinki*, which are four of the more common nototheniid species in McMurdo Sound and known prey of Weddell seals. DMSP concentrations were three times greater in these species compared to *D. mawsoni* (333 µmol kg^{-1}). The absence of DMSP does not mean females were not feeding on these species, but more likely DMSP had already cleared from seal plasma given its short half-life in humans and rats (Lee et al. 2004, Slow et al. 2004), and the fact that at 2 dpp, TMAO and AsB were measurable in all seals but DMSP was not detected. It is also possible that plasma DMSP was below the detection limit of the current LC-MS/MS assay and could not be accurately detected.

The presence of homarine in four out of nine females that fed during the study period provides evidence that these females were feeding on cephalopods. Previous diet studies confirm that Weddell seals do feed on octopods and squid in McMurdo Sound (Dearborn 1965, Castellini et al. 1992, Burns et al. 1998). Cephalopods appear to be the most likely source of plasma homarine since homarine was not detected in *P. antarcticum* and only in low amounts in *D. mawsoni* (25 µmol kg^{-1}) compared to millimolar concentrations in octopod and squid species (Chapter Four, Table 4.2). Even though plasma homarine and TMAO were found in all four seals and plasma concentrations largely tracked each other, females 40 and 819 contained large amounts of plasma homarine (12.2 and 7.5 µmol l^{-1}, respectively). The TMAO:homarine ratios in Figure 5.20 show that lactating Weddell seals acquire different

concentrations of homarine depending on the type of species they are eating, with the highest amounts of homarine coming from octopus and the lowest from fish. Two scenarios are therefore possible. One is that a seal feeds on two different prey types and acquires homarine and TMAO separately; while the second is that a seal acquires homarine and TMAO from a single prey source.

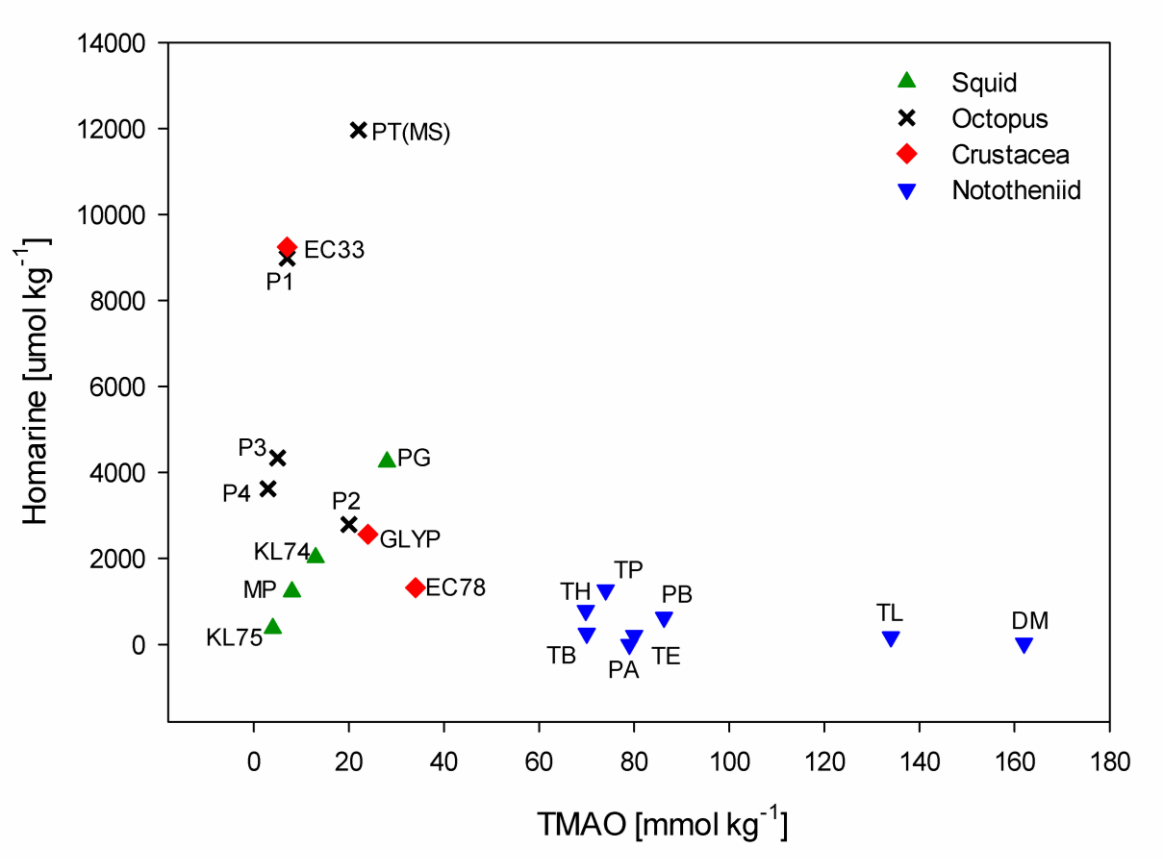


Figure 5.20. Plot of homarine *versus* TMAO in prey that may be eaten by lactating females in McMurdo Sound. Biomarker concentrations reported in Table 4.2, Chapter Four. Species abbreviations: **Nototheniids (blue triangles)** DM=*Dissostichus mawsoni*, PB=*Pagothenia borchgrevinki*, PA=*Pleuragramma antarcticum,* TB=*Trematomus bernacchii*, TE=*Trematomus eulepidotus*, TH=*Trematomus hansoni*, TL=*Trematomus lepidorhinus*, TP=*Trematomus pennellii*, **Crustaceans (red diamonds)** EC33 and EC78=*Euphausia crystallorophias*, GLYP=*Glyptontous antarcticus*, **Squid (green triangles)** KL74 and KL75=*Kondakovia longimana*, MP=*Mastigoteuthis psychrophila*, PG=*Pyschroteuthis glacialis*, **Octopus (black x's)** P1=*Pareledone* cf. *albimaculata*, P2=*Pareledone* cf. *aequipapillae*, P3=*Pareledone* cf. *charcoti*, P4= *Pareledone* cf. *Turqueti,* PT(MS)=*Pareledone turqueti* McMurdo Sound.

From the data listed in Table 4.2 (Chapter Four), we can also calculate the different numbers of fish that a female Weddell seal would have to eat in order to obtain a comparable amount of homarine absorbed after ingestion of octopus or squid in McMurdo Sound that contains *c.*

12 mmol kg^{-1} homarine. If we assume the mean mass of a single *P. turqueti* caught by a Weddell seal is 57 g (Xavier et al. 2002), the amount of homarine ingested with a single 57-g octopus is 683 µmol. For a Weddell seal to obtain the equivalent amount of homarine, it would have to catch and eat 8-9 *P. borchgrevinki* (total mass 1,330 g) with an average body mass of 133 g and homarine content of 600 µmol, or 3 *D. mawsoni* (total mass 25.2 kg) with a homarine content of 25 µmol kg^{-1} in its muscle tissue. The mean mass of toothfish caught by a Weddell seal is about 14 kg and the edible portion appears to be about 60% of this, or 8.4 kg (Calhaem and Christoffel 1969, Ainley and Siniff 2009). If a seal was to consume the larger squid *K. longimana* with an average mass of 4 kg (Xavier et al. 2002) and a homarine content of 1,200 µmol, then it would have to catch and eat 70 *P. borchgrevinki* and 20 *D. mawsoni* to obtain the equivalent amount of homarine.

The elimination of GB, TMAO, DMSP and AsB have been well described in human and laboratory animals (Vahter et al. 1983, Al Waiz et al. 1987, Mitchell et al. 1997, Lee et al. 2004, Slow et al. 2004), but nothing is known about their kinetics in marine mammals. AsB is retained in circulation for 3–5 days providing information on feeding *versus* fasting over a longer time period. AsB enters tissues as a GB analogue and has a long half-life in Weddell seals (Eisert et al. 2005). In contrast, TMAO in human and rat trials disappears from circulation with twenty four hours of feeding, providing information on recent food intake. However, this may not be the case for TMAO in marine mammals, or at least in Weddell seals. TMAO plasma concentrations continued to decline during the first seven days post partum and were still quantifiable after two weeks. TDR records show that these animals were not diving (except female 946 which started diving at 9 dpp) and therefore the delayed decay is not due to feeding. It is probably due to the high quantity of TMAO present in Antarctic fish (up to 162 mmol kg^{-1}, Chapter Four) and the greater quantity of fish consumption compared to human volunteers (Weddell seals can consume up to 200 *P. antarcticum* in a single feeding bout, Plötz et al. 2001). Randall et al. (1996) found that homarine is excluded from canine kidney cells and therefore homarine does not appear to distribute into tissues. It is likely that homarine is also quickly excreted *via* the urine, similar to TMAO. Unfortunately there are no data at present on the excretion kinetics of homarine.

Two compounds that are not synthesised by mammals that could be potential biomarkers are octopine and proline betaine. Octopine belongs to the group of amino acids called “opines”, which are the end products of anaerobic glycolysis in invertebrates (de Zwaan 1977) and is

primarily accumulated in the muscles of molluscs during short-term burst activity and thus it performs the equivalent role of lactate in mammalian muscle (Hochachka et al. 1977). Octopine has been identified in the squid *Nautilus pompilius* (Hochachka et al. 1977) and *Loligo vulgaris* (Grishaber and Gäde 1976) as well as in the scallop species *Pecten jacobaeus* (Grishaber and Gäde 1976) and *P. maximus* (Gäde et al. 1978). It is possible that octopine may be a more suitable biomarker than homarine for distinguishing between cephalopods and fish in Weddell seal diets. Proline betaine (*N*,*N*-dimethylproline or stachydrine) is an osmolyte that is accumulated in large quantities in response to drought or salinity stress by a number of plants (Ashraf and Foolad 2007) and is present in particularly high concentrations in citrus fruit and in some legumes (Slow et al. 2005). Extensive work has been carried out on proline betaine in common foods and plants, yet little is known about its presence in seafood. Proline betaine has been isolated from the adductor muscle of the fan-mussel *Atrina pectinata* (Hayashi and Konosu 1977), in the ovaries of the shellfish *Callista brevi* (Yasumoto and Shimizu 1977), and in the euryhaline mollusc *Elysia chlorotica* (Pierce et al. 1984). Yancey et al. (2010) have also quantified proline betaine in coral tissues. Thus, it is likely that proline betaine may be another suitable potential biomarker, similar to octopine, for distinguishing between invertebrates and fish in pinniped diets.

It was difficult to categorise a few of the seals as feeding because even though biomarker levels increased in mid- or late lactation, they never reached levels above the fasting limits. These results indicate that the seals could have been feeding but plasma biomarker concentrations were in decline by the time the seals were sampled. Biomarkers are a conservative estimate of feeding and the probability of detection increases with increased sampling frequency. However, any sampling needs to be balanced with the amount of disturbance, the effects on the animal, and logistical feasibility. Increases in plasma biomarker concentrations were concurrent with the onset of diving activity; however, this study shows that even though seals are diving they are not always feeding. Female 5746 made deep dives throughout mid and late lactation, but plasma biomarker concentrations never rose above the fasting limits. Female 819 started diving at 13 dpp but did not start feeding until some time between 19–24 dpp, and there was no evidence of feeding in female 515 after 18 dpp even though this seal continued to dive regularly and to considerably deep depths well into late lactation.

The present study focused on lactating females in McMurdo Sound, but elsewhere in the Southern Ocean, Weddell seals appear to forage on a greater diversity of prey than has been reported for McMurdo Sound (Plötz et al. 1991, Plötz et al. 2001, Lake et al. 2003) where their diet may change from one year to the next in relation to local food supply (Plötz et al. 1991). Further investigation on the biomarker composition of the prey and plasma of lactating females in these areas as well as males and juvenile animals, are required to test and refine the biomarker method. Further work is also needed on the excretion rates of biomarkers in pinnipeds, especially DMSP and homarine, and these studies can be carried out on captive animals. It is also essential to identify other compounds, in addition to octopine and proline betaine, which will help to fine tune the biomarker patterns observed between the major groups of Weddell seal prey.

Chapter Six

Proximate composition and energy density of important prey items to Weddell seals in McMurdo Sound and the Ross Sea, Antarctica

The Antarctic toothfish, *Dissostichus mawsoni*, in a holding tank at Scott Base, Antarctica. Photo provided by E. Neufeld, Gateway Antartica.

6.1 Introduction

Compared with temperate and tropical regions, piscivores in the Southern Ocean have access to relatively low prey species diversity. Benthic fish species, and especially those of the perciform suborder Notothenioidei, predominate in the fish fauna of the Antarctic continental shelf and upper slope (Eastman 2005). In the Ross Sea, notothenioids account for 76% of fish species diversity, 91% of species abundance and 92% of biomass, respectively (Eastman and Hubold 1999). Notothenioids are the most important food source for marine birds and mammals foraging on the continental shelf (La Mesa et al. 2004b, Smith et al. 2007). Among notothenioids, the family Nototheniidae is particularly speciose, with many benthic as well as benthopelagic or pelagic (*e.g.*, silversides, *Pleuragramma antarcticum*; subadult and adult Antarctic toothfish, *Dissostichus mawsoni*) and cryopelagic (bald notothen or "borchs", *Pagothenia borchgrevinki*) species.

In the Ross Sea, seals, birds and whales feed heavily on nototheniids, and a single species, *P. antarcticum*, can contribute a substantial portion to their diet (Ainley et al. 1984, Ainley et al. 1998, Burns et al. 1998, Cherel and Kooyman 1998, Ichii et al. 1998, Lauriano et al. 2007). For example, some studies suggest that Weddell seals in the Ross Sea feed predominantly on *P. antarcticum*, while others indicate greater diversity or emphasize the potential importance of other prey such as cephalopods and in particular the large Antarctic toothfish, *D. mawsoni* (Dearborn 1965, Calhaem and Christoffel 1969, Burns et al. 1998, Ainley and Siniff 2009). The contribution of *D. mawsoni* to Weddell seal diets has been difficult to quantify given that seals selectively ingest the soft tissue rather than diagnostic hard parts (such as otoliths and skeletal elements) and thus toothfish is unlikely to be detected using traditional methods of diet assessment.

Given the nutritional importance of notothenioids in sea bird and marine mammal diets, surprisingly little information is available on the nutrient composition of these fish. Lipid content of notothenioid tissues has been examined with regard to fish feeding ecology and buoyancy (Friedrich and Hagen 1994, Hagen et al. 2000) but it is whole-body lipid content that is important to piscivores that consume entire prey. As most, if not all Weddell seals appear to initiate feeding during lactation (Chapter Five), knowledge of the energy content of prey is essential to estimate food requirements associated with reproductive success. This information is also of importance for understanding the trophic relationships and energy and

nutrient fluxes within McMurdo and Ross Sea ecosystems. It is not known if the nutritional value, relative abundance, seasonal patterns of reproduction, or responsiveness to local environmental conditions (such as nutrient fluxes at the ice edge) of Weddell seal prey species impact the distribution or success of local breeding colonies of this predator, but it seems likely. If females need to forage in late lactation to prevent excessive depletion of body reserves, it stands to reason that the quality and abundance of local prey may be essential to sustaining lactation. However, in the absence of reliable data on nutrient composition of prey, it is hard to argue which prey species may have particular value to Weddell seals.

A wide array of fish species was obtained from within McMurdo Sound and from the Ross Sea, including benthic, pelagic and cryopelagic notothens (Nototheniidae), as well as lanternfishes (Myctophidae), a deepsea smelt (Bathylagidae) and the octopus *Pareledone turqueti*. Based on the findings of Chapter Five, cephalopods may be an important food item for Weddell seals in mid- to late lactation in McMurdo Sound.

6.2 Materials and methods

6.2.1 Sample collection

Prey captured from various locations within McMurdo Sound and the Ross Sea were used in this study. See Chapter Four, section 4.2.1 for details.

6.2.2 Proximate composition assays

In the laboratory, frozen specimens were partially thawed, blotted dry and weighed to the nearest 0.1 gram to determine wet mass; total length (TL) was measured to the nearest 0.1 cm (Table 6.1). Fish of <100 gram WM were pooled (n = 2–30 fish, depending on prey size), in addition to *Pareledone turqueti* (octopus) to obtain sufficient mass for all analyses reported herein. While still partially frozen, individuals or pooled specimens were homogenised whole (or after being cut into small pieces) with distilled water (added 1:1 m/m) in a commercial food blender. The wet homogenate was subsampled into aluminum pans, dried overnight in a forced convection oven at 56 ºC and ground using an electric kitchen grinder to a uniform consistency before being subsampled for all subsequent assays. The blender and grinder were cleaned and dried between samples.

Pooled and individual species were analysed in triplicate for each assay. Water content was determined by mass loss when separate 1 gram subsamples of wet homogenate were dried overnight in a forced convection oven at 56 ºC, and then for a further 2 hours at 100 ºC. Fat or neutral lipid was determined by overnight extraction (*c*. 16-18 hours) of 1 gram aliquots of dried, ground homogenate using petroleum ether in a Soxhlet fat extraction apparatus. Crude protein (CP) content [total nitrogen (TN) × 6.25] was determined by both CHN gas analysis and macro-Kjeldahl methods. For CHN gas analysis, 3-5 mg dried homogenate was subsampled into tin cups, dried at 56 ºC overnight and combusted at 950 ºC in a CHN elemental gas analyser (Model 2400, series 2, Perkin Elmer Co., Norwalk, CT, USA.) with supplemental oxygen boosts of 2 seconds. To validate the CHN method, a subset of samples (n = 23 of 34 samples) was also assayed using a conventional macro-Kjeldahl method. Subsamples of dried, ground homogenate (0.5–0.7 grams) were digested with 30 mL concentrated H_2SO_4 and catalyst (FisherTab™ CT-50 Kjeldahl tablets); the digests were rendered basic with sodium hydroxide (NaOH) and steam-distilled. Ammonia in distillates was titrated with hydrochloric acid (0.1 M HCl) to determine total nitrogen content of the sample. Ash was determined by combusting 0.5-1.0 gram subsamples of dried homogenate in porcelain crucibles in a muffle furnace (model FA17301, Thermolyne Sybron Corp, Dubuque, IA, USA). Temperatures were ramped up slowly from 100 ˚C to 400 ºC, and then set at 550 ºC for 5 hours. Gross energy content or energy density (ED, kJ g^{-1}) was determined for 0.4–0.6 grams of dried homogenate using an adiabatic bomb calorimeter (Model 1241, Parr Instruments, Moline, IL, USA) calibrated with pre-weighed benzoic acid tablets. Corrections were made for the energy equivalents of fuse wire combustion and acid production. Percent fat, CP, and ED are expressed per unit wet mass (% WM) unless otherwise stated. Ash is expressed as a proportion of fat-free dry mass (% FFDM).

6.2.3 Statistical analysis

Because pooled and individually analyzed fish of the same species represent different size classes (fish >100 gram mass were analysed individually, while smaller fish were pooled), box and whisker plots were used to compare individually analysed fish with pooled samples within species (*i.e.*, *Trematomus bernacchii* and *Pagothenia borchgrenvinki*) to monitor a possible effect of fish size on composition. Individual fish were mathematically pooled, a weighted average was determined for mass, and these values were compared to values of the individual fish and pooled samples.

Interspecies statistical comparisons of PC and ED were limited to three fish species which were analysed individually (*Trematomus hansoni* collected in 2007, *T. bernacchii* in 2007 and *P. borchgrevinki* in 2006 and 2007). A generalised linear mixed effects model was used to detect differences in PC and ED using the statistical package 'R' (version 2.10.1), using Tukey's test for *post ex facto* comparison ($\alpha = 0.05$) between groups.

6.3 Results

Within-sample coefficients of variation for the analyses were as follows (n = 34): moisture (0.5 ± 0.7%, range 0.1–3.6%), fat (0.8 ± 0.9%, range 0.03–3.1%), CP (2.0 ± 2.3, range 0.2–5.8%), ash (0.8 ± 0.5, range 0.1–2.8%) and ED (0.5 ± 0.6, range 0.1–3.3%).

Compositional data were obtained for 15 fish species from three families, including six species of *Trematomus*, single species of four other nototheniid genera, four species in two genera of myctophids, and a bathylagid (Table 6.1). The nototheniids were equally divided between McMurdo Sound and the open Ross Sea; the myctophids and bathylagid were collected near the Admiralty Seamounts, abyssal plain and Scott Seamounts in the Ross Sea.

Data were obtained from both pooled and individually analysed fish, with the latter representing a larger size class (individual mass > 100 grams). No compositional differences were apparent between the pooled or individually analysed fish on a wet or dry mass basis in those species for which this could be compared (Figures 6.1 and 6.2). Interspecies statistical comparisons of *T. hansoni*, *T. bernacchii* and *P. borchgrevinki* from McMurdo Sound showed that they did not differ in fat ($P = 0.630$), CP ($P = 0.277$) or ED ($P = 0.987$), but there were significant differences in ash content expressed as a proportion of FFDM ($F_{2,19} = 11.2$, $P < 0.001$). *T. bernacchii* had a significantly higher ash content (14.2% FFDM) than *P. borchgrevinki* (12.6% FFDM, $P < 0.001$) and *T. hansoni* (12.8% FFDM, $P = 0.015$).

Table 6.1. Proximate composition (% moisture, fat, crude protein, ash) and energy density (ED) of fish species collected from McMurdo Sound (MS) and the Ross Sea (RS). Fish were assayed individually (I) or as pooled samples (P). Values are mean ± SEM, expressed on the basis of wet (% WM) and dry mass (% DM). Ash was calculated as a proportion of fat-free dry mass (% FFDM).

Species	n	Method	Collection area	Length (cm)	Mass (g)	Water (% WM)	Fat (%WM)	Crude protein (%WM)	ED (kJ g^{-1} WM)
Nototheniidae									
Dissostichus mawsoni[a]	1	I[a]	MS	NA	983.2	68.6	15.1	15.4	9.4
Lepidonotothen squamifrons	5	P	RS	22.4 ± 31.7	291.0 ± 102.0	81.3	2.3	10.5	4.0
Pagothenia borchgrevinki 2006	2	P	MS	18.2 ± 0.3	88.7 ± 0.1	77.2	5.6	16.4	5.6
Pagothenia borchgrevinki 2006	4	I	MS	20.5 ± 2.6	125 ± 24.9	77.6 ± 3.1	4.8 ± 3.2	14.9 ± 2.4	5.3 ± 1.3
Pagothenia borchgrevinki 2007	4	I	MS	23.5 ± 2.7	162.6 ± 18.1	76.0 ± 2.5	5.2 ± 2.9	13.9 ± 1.8	5.7 ± 1.1
Pleuragramma antarcticum	15	P	RS	13.4 ± 2.1	23.7 ± 15.2	82.1	7.2	8.3	5.0
Trematomus bernacchii 2006	6	P	MS	14.6 ± 1.8	48.7 ± 6.1	78.3	2.9	14.5	4.7
Trematomus bernacchii 2007	7	P	MS	16.4 ± 2.5	77.1 ± 53.4	77.4	2.8	16.7	5.0
Trematomus bernacchii 2007	4	I	MS	18.9 ± 2.2	114.7 ± 23.3	76.2 ± 3.0	4.1 ± 3.3	15.2 ± 3.4	5.5 ± 1.3
Trematomus eulepidotus	5	P	RS	19.6 ± 3.1	121.8 ± 59.9	75.6	4.6	15.1	5.7
Trematomus hansoni	7	I	MS	21.1 ± 26.2	148.0 ± 53.2	76.7 ± 2.0	3.7 ± 2.5	15.4 ± 1.5	5.4 ± 0.9
Trematomus lepidorhinus	5	P	RS	27.4 ± 5.6	217.0 ± 102.4	71.3	9.4	15.1	7.7
Trematomus pennellii	2	P	MS	14.1 ± 1.6	46.1 ± 4.4	78.3	2.6	14.2	4.6
Trematomus scotti	5	P	RS	12.9 ± 0.7	13.4 ± 2.1	78.5	0.5	14.8	4.0
Myctophidae									
Electrona antarctica	30	P	RS	8.1 ± 1.0	7.4 ± 2.5	69.6	15.2	12.2	9.0
Electrona carlsbergi	15	P	RS	7.2 ± 0.6	5.5 ± 1.6	73.9	7.6	13.9	6.1
Gymnoscopelus braueri	5	P	RS	10.1 ± 0.7	9.0 ± 1.9	68.5	14.9	13.7	9.3
Gymnoscopelus nicholsi	5	P	RS	14.9 ± 0.7	37.0 ± 3.4	64.9	17.4	14.3	10.3
Bathylagidae									
Bathylagus antarcticus	5	P	RS	15.1 ± 2.0	38.6 ± 18.2	87.3	2.6	7.7	2.9
Octopodidae									
Pareledone turqueti	4	P[b]	MS	28.4 ± 1.9	N/A	84.3	8.3	10.8	3.4

Table continued on next page.

Species	Fat (%DM)	Crude protein (%DM)	Ash (%FFDM)	ED (kJ g^{-1} DM)
Nototheniidae				
Dissostichus mawsoni[a]	50.4	49.1	-	31.6
Lepidonotothen squamifrons	14.2	71.0	15.8	24.5
Pagothenia borchgrevinki 2006	25.7	63.7	12.8	25.7
Pagothenia borchgrevinki 2006	21.3 ± 5.4	66.4 ± 4.0	13.1 ± 0.3	25.1 ± 2.4
Pagothenia borchgrevinki 2007	22.1 ± 4.6	67.4 ± 4.0	11.2 ± 0.1	24.7 ± 1.7
Pleuragramma antarcticum	41.6	48.8	13.1	29.2
Trematomus bernacchii 2006	13.7	71.1	14.4	22.4
Trematomus bernacchii 2007	12.7	74.0	13.6	22.8
Trematomus bernacchii 2007	16.8 ± 6.1	69.5 ± 4.5	14.2 ± 0.3	23.3 ± 2.9
Trematomus eulepidotus	19.1	68.3	12.1	24.4
Trematomus hansoni	15.7 ± 3.7	71.3 ± 3.2	12.8 ± 0.1	23.6 ± 1.5
Trematomus lepidorhinus	32.9	53.2	12.4	27.1
Trematomus pennellii	12.6	71.4	15.7	22.1
Trematomus scotti	2.4	75.5	22.0	18.3
Myctophidae				
Electrona antarctica	51.4	40.8	15.8	30.4
Electrona carlsbergi	29.8	57.2	15.1	25.7
Gymnoscopelus braueri	49.4	43.8	15.7	30.9
Gymnoscopelus nicholsi	51.2	41.9	12.4	30.4
Bathylagidae				
Bathylagus antarcticus	21.9	64.9	16.8	24.8
Octopodidae				
Pareledone turqueti	52.9	69.0	24.3	21.9

[a] values are for a fillet of white muscle, [b] measurement of three *Pareledone*, one distintegrated on thawing and could not be accurately measured.

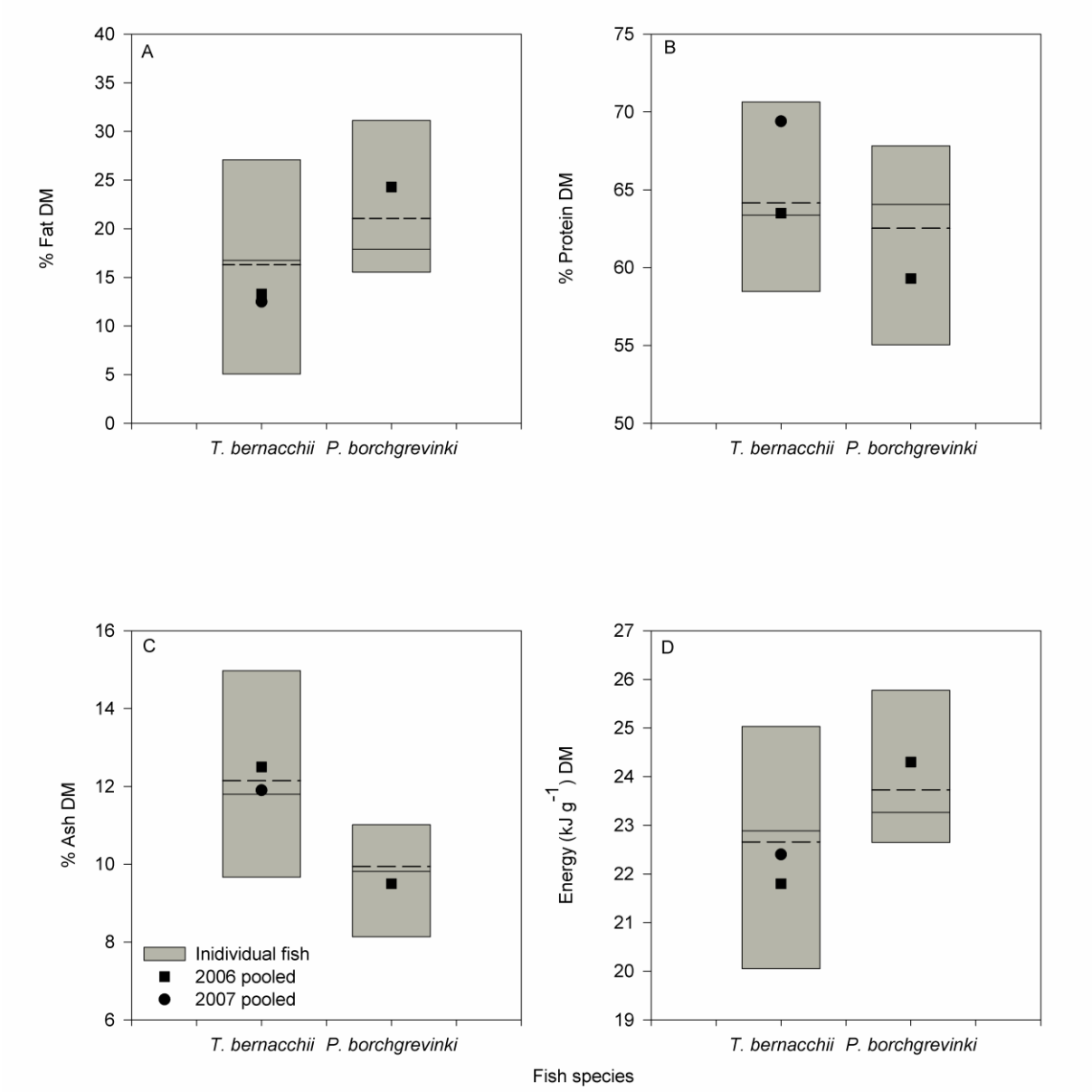


Figure 6.1. Box and whisker plots comparing individually analysed fish (*Trematomus bernacchii* and *Pagothenia borchgrevinki*) with pooled samples within species for % fat (A), % protein (B), % ash (C) and energy content (kJ g^{-1}) (D) on a on a dry mass basis. Shaded boxes represent individual fish [lower quartile (25%); upper quartile (75%); median (solid line); mean (dashed line)]. Black squares (2006) and circles (2007) indicate mean values of pooled fish.

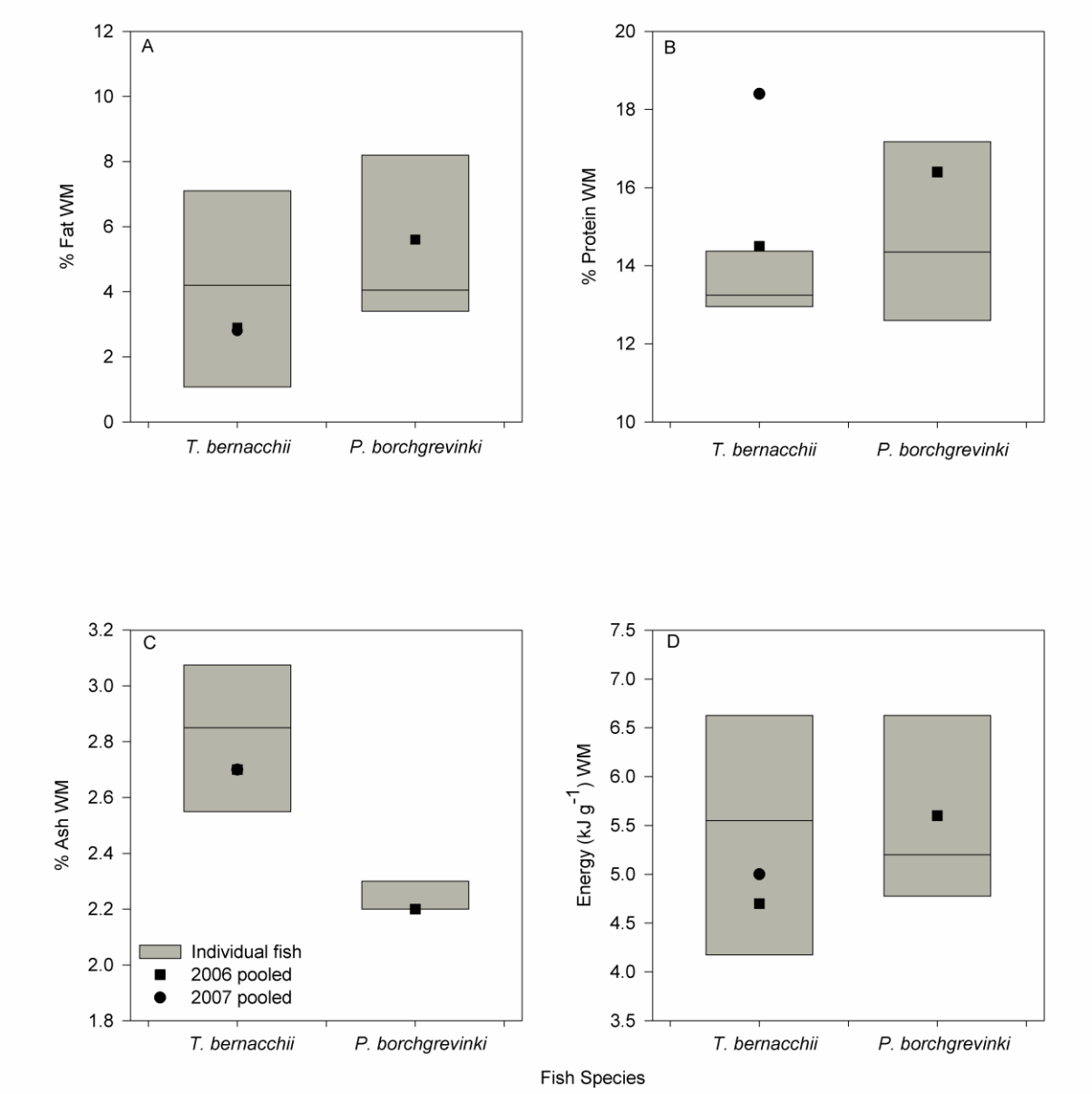


Figure 6.2. Box and whisker plots comparing individually analysed fish (*Trematomus bernacchii* and *Pagothenia borchgrevinki*) with pooled samples within species for % fat (A), % protein (B), % ash (C) and energy content (kJ g^{-1}) (D) on a wet mass basis. Shaded boxes represent individual fish [lower quartile (25%); upper quartile (75%); median (solid line); mean (dashed line)]. Black squares (2006) and circles (2007) indicate mean values of pooled fish.

Moisture content of whole fish was variable, ranging from a low of 64.9% WM (*Gymnoscopelus nicholsi*) to 87.3% WM (*Bathylagus antarcticus*, Table 6.1). Fat content was also variable, ranging from 0.5% WM (*Trematomus scotti*) to 17.4% WM (*Gymnoscopelus braueri*). Myctophids were particularly high in fat, while benthic *Trematomus* spp. were low in fat. Among nototheniids whole *Trematomus lepidorhinus* and

D. mawsoni muscle tissue had particularly high fat contents. The sample of *D. mawsoni* consisted of 900 gram of white muscle from the only toothfish caught in McMurdo Sound in 2007 (A. DeVries, personal communication). Among the nototheniids from McMurdo Sound, the sample of *D. mawsoni* fillet was highest in fat (15.1% WM) while *Trematomus pennellii* was lowest in fat content (2.6% WM).

Total nitrogen content estimated for the same samples by CHN and macro-Kjeldahl methods did not differ (paired t-test: t = 2.07, $P = 0.766$, $df = 22$); CP results from the larger CHN dataset are reported. CP content was relatively low for *P. antarcticum* (8.3% WM) and *B. antarcticus* (7.7% WM) while benthic *Trematomus* species (*e.g. T. bernacchii*) were high in CP (Table 6.1). Among the nototheniids, pooled *T. bernacchii* from 2007 was highest in CP. Ash content (% FFDM) was high in myctophids and the benthic *T. scotti*, and low for pelagic species such as *P. antarcticum*. Among nototheniids, the epibenthic species *Trematomus eulepidotus* and *T. lepidorhinus* were lowest in ash content on a FFDM basis (Table 6.1).

ED of whole fish varied more than three-fold, between 2.9 kJ g^{-1} WM (*B. antarcticus*) to 10.3 kJ g^{-1} WM (*G. nicholsi*). Fat content was the primary determinant of energy content and explained 90% of variation of ED between samples (Pearson correlation r = 0.96, $P < 0.001$). Myctophids were high in ED, as was *D. Mawsoni* muscle tissue. Among the nototheniids caught in McMurdo Sound, *T. pennellii* was lowest in ED (4.6 kJ g^{-1}, Table 6.1).

6.4 Discussion

This research provides important new information about the nutritional value of a range of known and potential prey species available to Weddell seals in McMurdo Sound and the Ross Sea. As fish were only obtained during the austral spring (McMurdo Sound) and summer (Ross Sea), these data provides a snapshot of nutrient composition at these times; it is not known whether any of the species studied undergo changes in fat and energy content in response to seasonal changes in productivity or as a function of reproductive effort or migratory pattern. A limitation is that pooled samples may disguise variation among individual fish associated with sex, reproductive status, body condition, age and body size. Tierney et al. (2002) found that smaller individuals tended to have higher energy content

while other studies report the opposite (Van De Putte et al. 2010). Lawson et al. (1998) found that energy density differed with size in some (*e.g.*, halibut) but not all (*e.g.*, Atlantic cod) species in the Northwest Atlantic. In this study, despite 2–3 fold differences in mass of individual fish for two species (bald notothen, *P. borchgrevinki*, and emerald notothen, *T. bernacchii*) for which there was both individual and pooled results, there was no apparent difference in energy density.

These results represent the first broad study of the nutrient composition and energy density of fish in the Ross Sea. Published data are available on the water, lipid and/or energy contents of the four myctophids of the genera *Electrona* and *Gymnoscopelus* at other locations: Bird Island (Clarke and Prince 1980), Croker Passage (Reinhardt and Vanvleet 1986), Kerguelen Plateau (Lea et al. 2002a, Connan et al. 2010), Macquarie Ridge (Lea et al. 2002a, Tierney et al. 2002), the Lazarev Sea (Van De Putte et al. 2006) and the Cosmonaut Sea (Van Putte et al. 2010) (Table 6.2). These studies agree with our findings (for fish of similar size class) in that these myctophids are high in fat (12–19% vs. 8–17% WM in this study) and energy (7–13 kJ g^{-1} vs. 6–10 kJ g^{-1} WM), although Tierney et al. (2002) reported a somewhat lower energy density (5.4 kJ g^{-1}) for *E. carlsbergi* at Macquarie Ridge. Other myctophid species in the Southern Ocean also have high fat and energy contents, with the possible exception of *Protomyctophium* spp. that contain 4–10% WM fat and 4–7 kJ g^{-1} (Donnelly et al. 1990, Lea et al. 2002a, Tierney et al. 2002, Connan et al. 2010). In general, myctophid are lipid- and energy-rich prey, due to accumulation of triacylglycerols (*e.g.*, *E. carlsbergi*, *G. nicholsi*) and wax esters (*e.g.*, *E. antarcticus*, *G. braueri*) (Phleger et al. 1997, Phleger et al. 1999, Connan et al. 2010). It is not known if myctophid species high in wax esters are more difficult to digest (Place 1992), although some sea birds and whales appear to have compensatory mechanisms to allow wax ester utilisation (Place 1992, Nordoy 1995).

Table 6.2. Comparison of mean water (%), lipid (%) and energy (kJ g^{-1}) concentrations in fish of similar size from this study with other regions of the Southern Ocean.

Year	Region	N	Length (cm)	Water content (%)	Energy content WM (kJ g^{-1})	Energy content DM (kJ g^{-1})	Lipid content WM (%)	Lipid content DM (%)	Source
	Electrona antarctica								
1982	Croker Passage	-	not given	-	-	-	-	56.6	Reinhardt & Van Vleet 1986[a]
1986	Weddell/Scotia Sea	8	7.5 – 9.9	68.1			11.78	36.9	Donnelly et al. 1990
1998	Iles Kerguelen	5	6.5 ± 8.6	60.8	13.3	34.3			Lea et al. 2002
1999	Macquarie Ridge	6	> 5.5	66.3	10.6	31.4	-	-	Tierney et al. 2002
2004	Lazarev Sea	14	6.9 – 10.2	66.7	10.3	30.7	-	-	Van de Putte et al. 2006
2010	Cosmonaut Sea	5	7.8 – 10.2	71.1	8.44	29.1	-	-	Van de Putte et al. 2010
2008	Ross Sea	30	6.4 – 9.9	69.6	9.0	29.7	15.2 -	50.2	this study[b]
	Electrona carlsbergi								
1976	Bird Island	3	not given	71.2	6.6	22.8	-	-	Clarke & Prince 1980[c]
1998	Kerguelen Plateau	6	8.5 ± 3.6	67.0	8.6	25.9	12.2	37.0	Lea et al. 2002 [d]
1999	Macquarie Ridge	6	not given	76.7	5.1	21.7	-	-	Tierney et al. 2002
2008	Ross Sea	15	6.1 – 8.6	73.9	6.6	25.2	7.6	29.2	this study
	Gymnoscopelus nicholsi								
1998	Kerguelen Plateau	1	12.8	66.8	9.8	28.0	18.0	54.2	Lea et al. 2002
2008	Ross Sea	5	14.5 – 15.8	64.9	10.4	29.6	17.5	49.8	this study
	Gymnoscopelus braueri								
1976	Bird Island	3	not given	66.10	9.06	27.3	-	-	Clarke and Prince 1980
1999	Macquarie Ridge	6	6.0 – 10.4	66.1	9.9	29.2	-	-	Tierney et al. 2002
2004	Lazarev Sea	20	6.4 – 13.2	69.9	8.7	29.4	-		Van de Putte et al. 2006
2008	Ross Sea	5	9.5 – 11.2	68.6	9.4	29.8	-	-	this study
	Bathylagus antarcticus								
1982	Croker Passage	-	not given	-	-	-	-	23.2	Reinhardt & Van Vleet 1986
1986	Weddell/Scotia Sea	1	12.5 – 14.9	89.2			0.9	8.3	Donnelly et al. 1990
1999	Macquarie Ridge	6	> 12.1	80.5	4.2	21.9	-	-	Tierney et al. 2002
2004	Lazarev Sea	12	3.6 – 12.3	85.6	2.92	20.4	-	-	Van de Putte et al. 2006
2008	Ross Sea	5	12.5 – 16.6	87.3	3.0	23.5	2.6 -	20.8	this study

Year	Region	N	Length (cm)	Water content (%)	Energy content WM (kJ g^{-1})	Energy content DM (kJ g^{-1})	Lipid content WM (%)	Lipid content DM (%)	Source
	Pleuragramma antarcticum								
1982	Croker Passage	-	not given	-	-	-	-	47.7	Reinhardt & Van Vleet 1986
1991	Weddell/Lazarev Sea	18	12.0 – 19.0	78.3	-	-	10.4	47.0	Friedrich & Hagen 1994[e]
1991	Weddell/Lazarev Sea	4	12.0 ± 2.2	-	-	-	-	37.7	Hagen et al. 2000[f]
2010	Cosmonaut Sea	2	> 10.5	70.2	7.6	25.5	-	-	Van de Putte et al. 2010
2008	Ross Sea	15	9.0 – 19.1	82.2	5.1	28.4	7.2	40.4	this study
	Trematomus lepidorhinus								
1991	Weddell/Lazarev Sea	35	13.0 – 24.0	75.1	-	-	5.2	20.8	Friedrich & Hagen 1994
1991	Weddell/Lazarev Sea	4	16.0 ± 0.8	-	-	-	-	23.2	Hagen et al. 2000
2008	Ross Sea	5	21.7 – 30.7	71.3	7.8	27.1	9.4	32.9	this study

Lipid extraction methods
[a] modified Folch et al. 1956
[b] Soxhlet petroleum ether (neutral lipids)
[c] Bligh and Dyer 1959
[d] modified Bligh and Dyer 1959
[e] modified Folch et al. 1956
[f] modified Folch et al. 1956

Unfortunately there are few published data on nototheniids. Lipid content of *P. antarcticum* is reported as 10.4% WM and 38–48% DM in the Weddell/Lazarev Sea and Croker Passage (Reinhardt and Vanvleet 1986, Friedrich and Hagen 1994, Hagen et al. 2000) as compared to our values of 7.2% WM and 41.6% DM (Table 6.1). This species has an energy density of 26 kJ g^{-1} dry mass in the Cosmonaut Sea (Van de Putte et al. 2010); we measured 28 kJ g^{-1}. In the Weddell/Lazarev Sea *T. lepidorhinus* has been found to contain 5.2% lipid on a WM basis and 21–23% lipid on a DM basis (Friedrich and Hagen 1994; Hagen et al. 2000), compared to 9.4% and 32.9% in this study. The single *D. mawsoni* muscle fillet that we analysed was high in lipid and energy (Table 6.1), presumably due to deposition of triacylglycerols; nototheniids do not deposit wax esters in significant amounts (Eastman 1993). The fat content reported here is within the range (6.6–23.0% WM) reported by Clarke et al. (1984) for white muscle of *D. mawsoni* caught in McMurdo Sound.

The bathylagid *B. antarcticus* has been reported to contain as little as 0.9% WM lipid in one study (Donnelly et al. 1990), but we found more than twice this amount (2.6% WM, equivalent to 21% on a DM basis). Reinhardt and Van Vleet (1986) reported 23% DM lipid in this species (Table 6.2). The energy density of *B. antarcticus* has been reported as 2.9–3.9 kJ g^{-1} WM (Tierney et al. 2002; Van de Putte et al. 2006), similar to our measurement of 3.0 kJ g^{-1} WM. Thus, available published data for this species from various regions around Antarctica are in reasonable agreement with our results for fish similar in length, although the comparisons are confounded by differences in analytical methodology. We assayed neutral lipids by ether extraction, whereas other investigators have typically used modified Bligh and Dyer and Folch procedures. The latter two methods extract polar lipids in addition to neutral lipids, but do not necessarily achieve complete extraction, especially at high lipid concentrations (Iverson et al. 2001).

The results suggest an effect of both phylogeny and life history on fish proximate composition in the Antarctic. On the one hand, myctophids were high in fat and energy both in our study and in prior reports from the Southern Ocean. Myctophids are also high in fat and energy in the subarctic Pacific, but not in the tropical Pacific (Seo et al. 1996, Saito and Murata 1998). Nototheniids were much more variable in composition, which may relate to habitat use. Notothenioids radiated from a benthic habitat when invading Antarctic waters which accounts for the lack of swim bladders, even in pelagic species such as silversides, *P.*

antarcticum, and Antarctic toothfish, *D. mawsoni* (Eastman 1993, Eastman 2005). These species are thought to achieve near neutral buoyancy by various morphological features, including accumulation of lipids in tissues (Eastman 1985a). High lipid accumulation is apparent in *D. mawsoni* (Table 6.1) and to a lesser extent in *P. antarcticum* (Table 6.1) which has subcutaneous lipid sacs along the sides of the body (Eastman 1985a). The cryopelagic *P. borchgrevinki* that associates with the underside of sea ice is not particularly high in lipids (4.8–5.6% WM), but it is considered more buoyant than benthic notothenioids (Eastman and Devries 1982). It is intriguing that *T. lepidorhinus* contained 9.4% lipids on a wet mass basis. This is consistent with evidence that *T. lepidorhinus* feeds away from the bottom, especially on pelagic amphipods, and thus may need to be more buoyant than other strictly benthic *Trematomus* species. However, Friedrich and Hagen (1994) and Hagen et al. (2000) reported somewhat lower lipid levels for this species, albeit from a different region of the Antarctic. Benthic species examined in this study (*Lepidonotothen squamifrons*, *T. bernacchii*, *T. hansoni*, *T. pennellii*, and *T. scotti*) had particularly low lipid (0.5–4.1% WM) and energy densities (4.0–5.5 kJ g^{-1} WM) among the nototheniids that were analysed (Table 6.1). This is not surprising, given that benthic species should have negative buoyancy and fat causes an increase in buoyancy. Benthic nototheniids typically have a higher proportion of body mass as skeleton which is high in ash (Eastman 1993) and whole body ash was high (14–21% FFDM) in the benthic species we examined. *Trematomus* species also tend to be generalist predators that feed mainly on low-energy benthic organisms (Montgomery et al. 1993, Vacchi et al. 1994), and thus their fat intake may be limited.

High lipid, high energy foods may be beneficial to sea birds and marine mammals, especially when recovering from periods of fasting or during reproduction (Boness and Bowen 1996). For large predators, *D. mawsoni* appears to be the highest quality fish prey item by virtue of its large size and high energy content, and is consumed both by Weddell seals and some killer whales, *Orcinus orca* (Calhaem and Christofell 1969, Lauriano et al. 2007, Ainley and Siniff 2009). Lactating Weddell seals initiate feeding during lactation (Sato et al. 2002, Eisert et al. 2005, Chapter Five) and rely on local prey resources available in the vicinity of breeding colonies. Testa et al. (1985) propose that Antarctic toothfish numbers are depressed near breeding concentrations in McMurdo Sound. Nutritional limitation due to insufficient quality or quantity of prey may result in reduction of maternal milk production or early weaning, with consequent reductions in pup survivorship (Proffitt et al. 2008). From the data listed in

Table 6.1, when we calculate the different numbers of prey that a Weddell seal would have to eat in order to obtain a comparable energy return to that of eating one toothfish, one can see why toothfish may be such an attractive prey choice. The mean mass of toothfish caught by a Weddell seal is about 14 kg and the edible portion appears to be about 60% of this, or 8.4 kg (Calhaem and Christoffel 1969, Ainley and Siniff 2009). For a Weddell seal to obtain the equivalent metabolisable energy to eating an average toothfish, it would have to catch and eat 672 *P. antarcticum* with a mean body mass (BM) of 24 g (n = 322 if mean BM = 50 g; Ainley and Siniff 2009), 110 *P. borchgrevinki* (BM = 133 g) or 220 *T. bernacchii* (BM = 76 g). While it is feasible for Weddell seals to ingest large numbers of swarming fish during a feeding bout (Plötz et al. 2001), this is improbable in the case of solitary fish or fish occurring at low densities.

Subadult and adult toothfish are too large for avian and many other predators, but the smaller *P. antarcticum* is both abundant and relatively rich in lipids compared with other nototheniids. *P. antarcticum* occupies a critical role in the food web of the Ross Sea. It is the main fish prey for emperor and Adélie penguins (Ainley et al. 1998, Cherel and Kooyman 1998) as well seabirds (Ainley et al. 1984). It is thought to be predominant in the diet of Weddell seals and is also a major food source for toothfish (Eastman 1985b). The predominance of *P. antarcticum* in predator diets is undoubtedly related to its abundance (Dewitt 1970) and occurrence as a clustered resource, as *P. antarcticum* is a schooling fish (Fuiman et al. 2002). A large number of individuals of this species (n > 200) may be consumed by individual Weddell seals in a single feeding bout (Plötz et al. 2001).

The combination of low energy content, small-medium body size and scattered distribution in solitary or small aggregations (Bill Davison, University of Canterbury, personal communication) implies that benthic *Trematomus* species may not be optimal prey resources for avian or mammalian piscivores in the Ross Sea, even though *Trematomus* remains have been found in stomach and/or faecal samples from Weddell seals (Burns et al. 1998) and emperor penguins (Cherel and Kooyman 1998) and may represent a significant component to Weddell seal diets in the Weddell Sea, at least in some years (Plötz et al. 1991). By contrast, the high lipid and energy content of myctophids suggest that these could have greater dietary importance in the Ross Sea than currently recognised. However, diet data for marine piscivores is limited outside of McMurdo Sound and myctophids are mesopelagic in waters of the continental slope and beyond (Eastman and Hubold 1999). Certainly myctophid

species are an important diet component for Weddell seals inhabiting sub-Antarctic islands (Casaux et al. 1997, Casaux et al. 2009) as well as for southern elephant seals (Cherel et al. 2008), Antarctic fur seals (Green et al. 1991, Lea et al. 2002b, Staniland et al. 2010), penguins (Hindell 1989, Hull 1999) and seabirds (Connan et al. 2007).

The biomarker data in Chapter Five suggests that females may feed on cephalopods during lactation. Squid species including *Gonatus antarcticus*, *Kondakovia longimana*, *Mastigoteuthiid* and *Brachiotuethiid* spp. as well as unidentified octopods have all been found in Weddell seals faeces and stomachs (Dearborn 1965, Testa et al. 1989, Burns et al. 1998). Castellini et al. (1992) also observed a Weddell seal feeding on the squid *Psychroteuthis glacialis*. To date, proximate composition data for cephalopods in McMurdo Sound is only available for the octopus *Pareledone turqueti* (this study). *Pareledone turqueti* is higher in fat content (8.4% WM) compared to the majority of fish analysed in this study (Table 6.1). For a Weddell seal to obtain the equivalent metabolisable energy to eating an average toothfish and other fish species (see above), it would have to catch and eat 235 *P. turqueti* with a mean BM of 57 grams (body mass data from Xavier et al. 2002). The squid species *G. antarcticus* and *K. longimana* are larger (mean body mass 218 and 4173 grams, respectively) than *P. turqueti*, and assuming an equivalent fat content to *P. turqueti*, Weddell seals would need to consume 61 *G. antarcticus* and 3 *K. longimana* (body mass data from Xavier et al. 2002). From this data one can see why cephalopods might be an attractive food choice and preferable to some fish species; more information is needed on their nutritional value. Unfortunately, the distribution of cephalopod fauna in the Ross Sea and McMurdo Sound is poorly known, so it not clear what species are available to Weddell seals in the vicinity of breeding colonies.

In conclusion, the results indicate that there are large differences in the quality of prey potentially available to Weddell seals in the Ross Sea. More research is needed on seasonal, reproductive, geographic, body size and other factors that may affect nutrient composition of Antarctic prey, and also to expand coverage to include other unique Antarctic fish taxa. It may also be important to focus sampling at different locations and seasons at various seal breeding colonies (*e.g.*, Terra Nova Bay, Cape Adare).

Chapter Seven

Summary and Directions for Future Work

Juvenile Weddell seal. Photo by W. Hood, Auburn University.

7.1 Summary of thesis

A central focus for this thesis was on the application of osmolytes and their analogues as *dietary biomarkers* to determine the onset of feeding and prey preferences in lactating Weddell seals (Chapters Four and Five). A second focal area of research was on the development of analytical methodologies for the detection of biomarkers in various biological matrices which included plasma, serum, prey tissues and whole prey (Chapters Two and Three). This final chapter reviews the key findings from the research with regard to the six hypotheses set out in Chapter One, section 1.6. Directions for future work are also discussed.

Hypothesis 5: Proton NMR and LC-MS/MS assays will be able to detect and measure dietary biomarkers in seal plasma and prey.

To assess the potential use of TMAO, AsB, GB, homarine and DMSP as species-specific biomarkers, it was first necessary to optimise an assay so that these compounds could be measured in various prey items of the Weddell seal. In Chapter Two, an ^{1}H NMR assay was developed by modifying procedures used by Lee et al. (2004, 2006) and Slow et al. (2004) for measuring betaines in urine. ^{1}H NMR was found to be an effective method for measuring and quantifying TMAO and GB within a 5 minute run time for each sample, but was inadequate to detect low concentrations of AsB, homarine and DMSP. This technique was also time consuming for large batches of samples since the instrument was not equipped with an autosampler. However, sample preparation was minimal and problems with sensitivity and sample throughput can be overcome in future studies by employing an instrument with greater magnetic strength, improved probe technology, and an autosampler which could make ^{1}H NMR the method of choice for future dietary biomarker studies.

An LC-MS/MS assay to quantify TMAO, AsB, GB, homarine and DMSP in prey and seal plasma was developed by modifying an assay published by Holm et al. (2003). Unfortunately, there were a number of problems that were encountered with the multi-user LC-MS/MS instrument that was used. The biggest issue was drift in the MS signal response after runs that injected raw urine into the instrument, which made it necessary to include additional control samples to correct for this. However, even with these issues sample

preparation was simple, derivitisation was not required as is the case for HPLC and GC, and DMSP, homarine and AsB were detected and measured in a number of prey samples that could not be detected using ^{1}H NMR. All of the analytes except for DMSP were detected in seal plasma and the LC-MS/MS results for TMAO and AsB in both prey and plasma correlated well with previously obtained results (Eisert and Oftedal unpublished data). This shows that the assay is valid, but any future studies should employ a more dedicated instrument with greater mass transition capabilities.

Hypothesis 1. Some but not all female Weddell seals feed during lactation.

Hypothesis 2. If females are feeding their plasma will contain the biomarkers TMAO, AsB, homarine, DMSP or GB.

Hypothesis 3. Measuring biomarkers in Weddell seal prey will tell us what the seals are feeding on if it can be shown that different prey types contain characteristic/specific biomarkers.

Hypothesis 4. Measuring several different biomarkers reduces the risk of failing to detect food consumption.

Foraging by female Weddell seals during lactation is the subject of ongoing debate, but the results presented in Chapter Five offer more conclusive evidence that females do feed (75% of females in this study) through the detection of three out of the five biomarkers in their blood. One of the main findings to emerge from this thesis was the presence of plasma homarine in four out of the eight females that fed during lactation. This suggests that females are feeding on squid and/or octopods in McMurdo Sound. Interestingly, none of the studies that have utilised animal-borne video or still cameras (Davis et al. 1999, Davis et al. 2003, Fuiman et al. 2007) have documented Weddell seals preying on cephalopods even though direct observations and the presence of beaks in faeces and stomachs provides clear evidence of cephalopod consumption by Weddell seals (Dearborn 1965, Burns et al. 1998). This is mainly due to the fact that the studies by Fuiman and Davis have been of seals at isolated holes away from breeding colonies. The foraging and diving behaviour of these seals does not represent the typical behaviour of lactating females. Additionally, these isolated holes were over deeper water, and there may be differences in the habitat under the isolated hole compared to holes closer inshore near breeding colonies.

The cluster analyses in Chapter Four showed a clear separation in biomarker content between the different groups of prey (*i.e.*, fish *versus* cephalopods). However, the contribution of individual species to TMAO, AsB and homarine concentrations found in plasma could not be determined at this time (Chapter Five). The biomarker method at present uses a limited number of feeding markers. This "biomarker library" needs to be expanded to other betaines and related compounds such as octopine and proline betaine, which are potentially more taxon specific than those tested in this thesis. Other potential prey species of Weddell seals that are present in McMurdo Sound and the Ross Sea need to be analysed for TMAO, GB, AsB, homarine and DMSP, while specimens lower in the food chain (*e.g.*, pteropods, copepods) are also needed for a better understanding of how osmolytes transfer through the food web. The number of individuals within each prey species also needs to be increased. Bryan et al. (1995) isolated the compound pteroenone from the pteropod *Clione antarctica* in McMurdo Sound. Even though *C. antarctica* doesn't appear to be a food item for fish, it is possible that other compounds like pteroenone are specific to Antarctic invertebrates and may also act as biomarkers of feeding. Another source of biomarkers might come from natural products, metabolites which have potential antibiotic and anticancer properties (Kwon et al. 2006, Blunt et al. 2012), and have been isolated from a number of marine organisms. This warrants further investigation.

There are a number of difficulties that constrain a study that is carried out in a remote environment on protected animals. The majority of blood samples were collected from females over the entire lactation period (38 days). However, it was not possible to capture some individuals multiple times (as in the case of female 1043) during each lactation stage and on a few occasions blood samples were taken 7-8 days apart. An opportunity to detect a feeding event may have been missed. If females are feeding, the frequency of sampling needs to be balanced so that shifts in diet between lactation stages can be monitored. Additionally, by the end of lactation when sea-ice starts breaking up, females start to move away from the colony and are no longer easily accessible for capture. It would also be beneficial to collect blood samples as soon as females haul out on the ice, as TMAO and AsB concentrations were already declining at the first collection date (2-3 dpp) and there would be a greater probability of detecting homarine or DMSP. However, this could cause stress to the mother and a two day period after giving birth is necessary for the mother-pup bond to form.

Hypothesis 6. If lactating Weddell seals are feeding, they are selectively feeding on energy rich prey.

If the number of toothfish is depressed near breeding colonies, it is plausible that females may in fact be targeting cephalopods. Octopus, at least *Pareledone turqueti*, are more nutritional than *Pagothenia borchgrevinki* and *Trematomus bernacchii* (Chapter Six) and this may also provide an energetic basis for targeting these species. Unfortunately, the distribution and abundance of cephalopods and the types of species present within McMurdo Sound are not well known, and this warrants further investigation. Given that Weddell seals need to consume significantly more of other prey types to obtain the equivalent amount of energy from that of one toothfish, a dietary method for detecting toothfish consumption is necessary, especially if females need high quality prey to complete lactation successfully.

7.2 Directions for Future Work

The biomarker method is in its early stages of development and a better understanding of the elimination kinetics of TMAO, AsB, GB, homarine and DMSP in pinnipeds is necessary for future studies. This information is not available for Weddell seals as this requires controlled feeding trials on captive animals and no Weddell seals are held in captivity. Therefore, at present, the reliability of the biomarker method to detect dietary preferences among individuals, or to estimate prey contributions in the diet, must be validated with diet estimates from other methods such as faecal analysis.

This thesis did not examine the relationship between food intake and milk quality which could have a profound impact on the reproductive performance of Weddell seals. Most of the research on the foraging behaviour in lactating females has been conducted in McMurdo Sound, which in comparison to other parts of the Antarctic represents an ecologically depauperate environment and therefore may not be representative of Weddell seal habitat elsewhere in the Antarctic, given that this species has a circumpolar distribution. This situation should favour larger and older females who can store larger amounts of fat and nutrients in their bodies and that employ a capital breeding strategy (Chapter One). Interestingly, the females who started diving and feeding the earliest (946 and 5891) were amongst the oldest and heaviest females studied, contrary to what might be expected. The biomarker method would be well suited towards studying the foraging strategies of lactating

females in other regions of the Antarctic (Weddell Sea and sub-Antarctic islands) who have access to a more diverse prey base and where species-specific biomarker patterns might be more distinct.

Future work should also explore the distribution of biomarkers in milk and the transfer of biomarkers in milk between mother and pup. The detection of biomarkers in pup plasma would provide confirmation of independent foraging by pups.

The examination of the nutritional quality (*i.e.*, vitamins, minerals) of prey items will also allow further insight into the prey preferences of lactating Weddell seals. It would also be beneficial to determine whether any of the prey species studied undergo changes in fat and energy content over the course of the lactation period, and whether the quality and amount of nutrients available affects Weddell seals at critical points during lactation.

The biomarker method is well suited for blood samples and therefore can be applied not only to other pinniped species but to seabirds, including penguins. This method could also be adapted for urine. However, collecting urine samples from free-ranging marine mammals may not be logistically feasible. AsB distributes into mammalian tissues, but since TMAO, and possibly homarine, do not, the biomarker method may not be applicable to studying the foraging behaviour of cetaceans. To explore this further, future research should include screening for and comparing biomarker concentrations in tissue or blubber biopsies.

7.3 Conclusion

The Erebus Bay Weddell seal population has provided a platform for researchers over the last forty years to study the foraging habits of females over the lactation period. The results from this thesis demonstrated that biomarkers and TDRs are complementary methods and when combined are a powerful tool for investigating the feeding habits of a top trophic predator. Although the biomarker method at this stage cannot identify individual species and sizes of fish being eaten, elaboration of this methodology will ensure that the biomarker method joins other standard tools for investigating diet and trophic relationships amongst a wide range of marine taxa.

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Appendix A

Arsenobetaine drift correction in prey samples

The drift in signal response during the course of the analytical run was considerable for AsB, as evident from the change in signal response between the first and later calibrations. There was no further drift between the second and third calibrations, so these data were pooled (Figure A1.1).

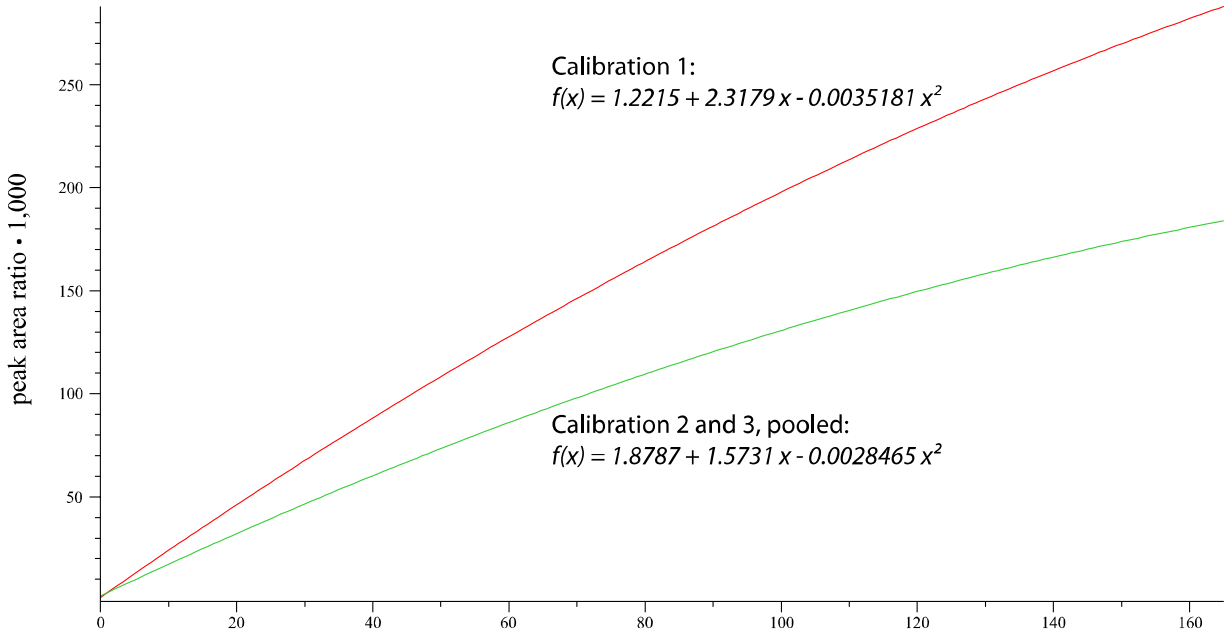

Figure A1.1. Change in AsB signal response (external calibration function) between the initial (1) and later (2, 3) calibrations.

The drift in signal response with time was not linear but varied with signal intensity, as becomes apparent when the drift (ratio of final and initial calibrations) is plotted against the initial signal intensity (peak area ratio; see Figure A1.2). It follows that the signal response of AsB during the run varied both with time and with concentration of AsB in the sample. Total drift in the signal (peak area ratio) *versus* initial signal was derived empirically by modelling the change in the signal from calibration 1 to calibrations 2 and 3 and can be described as (Figure A1.2):

$$f(x) = 0.753\, e^{-0.21}x - 0.75 \cdot 10^{-4}\, x + 0.635 \qquad Eq.\ 1$$

Change in the signal with time at a given concentration follows an exponential decay curve of the form:

$$g(t) = y_0 + a \cdot e^{-kt} \qquad \textit{Eq. 2}$$

$$g(0) = y_0 + a \qquad \textit{Eq. 2a}$$

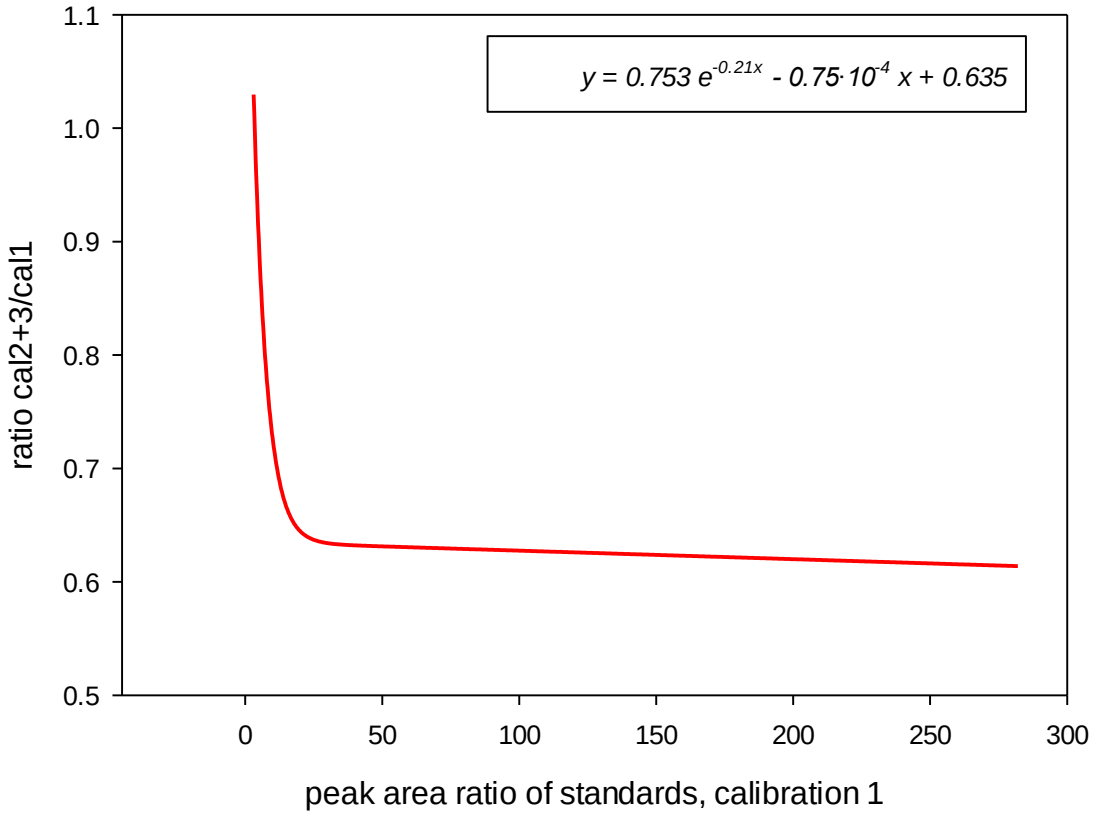

Figure A1.2. The amount of drift (ratio of signal response of final *versus* intial calibration) varies with size of the signal.

Because samples are injected into the instrument at regular intervals, vial sequence number (*t*) was used as a proxy for time (Figure A1.3). An exponential decay curve was modelled for each QC (25 and 80 µM) and standard (20 and 80 µM) sample separately to derive the initial signal value (*Eq. 2a*), and peak area ratios were normalised by dividing them by this initial value, *g(0)*. An exponential decay function was then fitted to the pooled normalised data to derive an estimate of *k*, the decay constant for the relative change of signal with time (Figure A1.3).

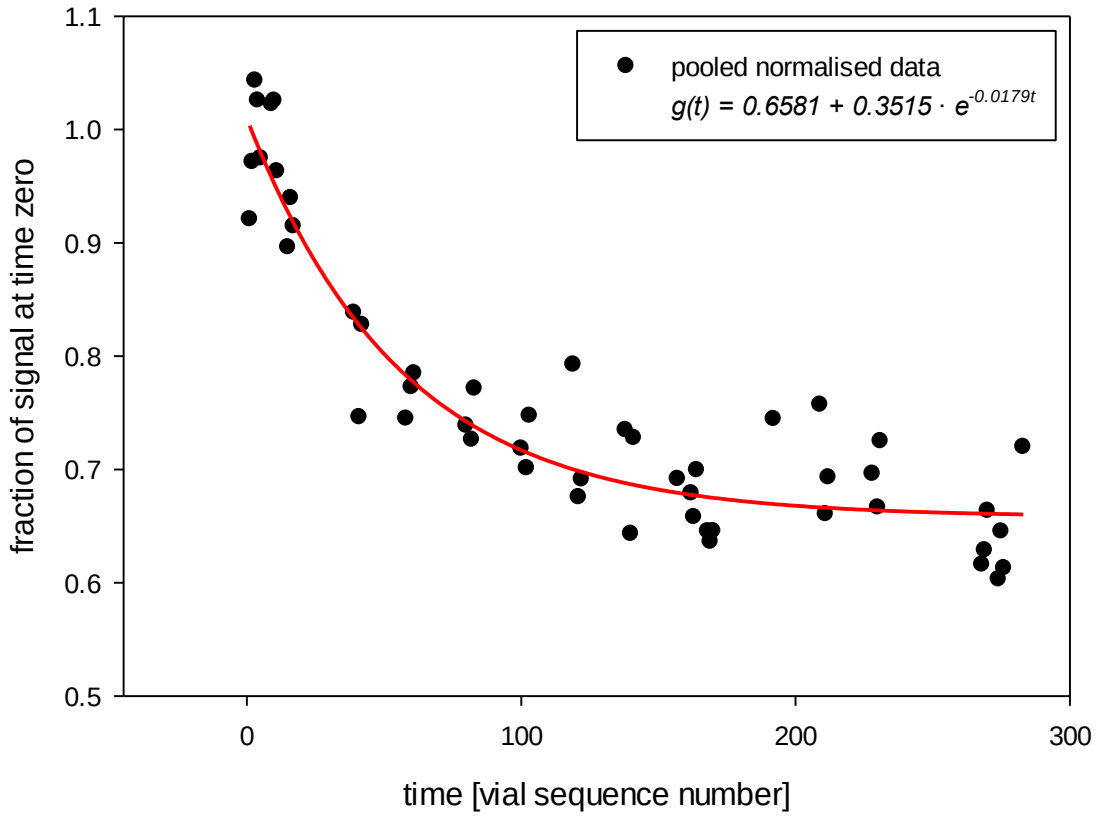

Figure A1.3. Pooled fractional drift in the normalised signal response of QC samples.

A drift correction factor f_c was modelled as the function of two independent variables, time (vial sequence number, t) and peak area ratio (x) so that:

$$f_c(x,t) = f(x) + [1 - f(x)] \cdot e^{-kt} \quad \text{Eq. 3}$$

$$f_c(x,0) = 1 \quad \text{Eq. 3a}$$

$$f_c(x,n) = f(x) \quad \text{Eq. 3b}$$

Where n is the total number of vials in the analytical run. The model (Figure A1.4) was parameterised by fitting it to the actual observed drift in QC samples. Measured peak area ratios for sample replicates were divided by the drift correction factor f_c to obtain the corrected peak area ratio. Corrected peak area ratios for calibration standards (Figure A1.5)

were used to derive a pooled calibration function to calculate concentration from the corrected peak area ratio of unknown samples (Figure A1.6).

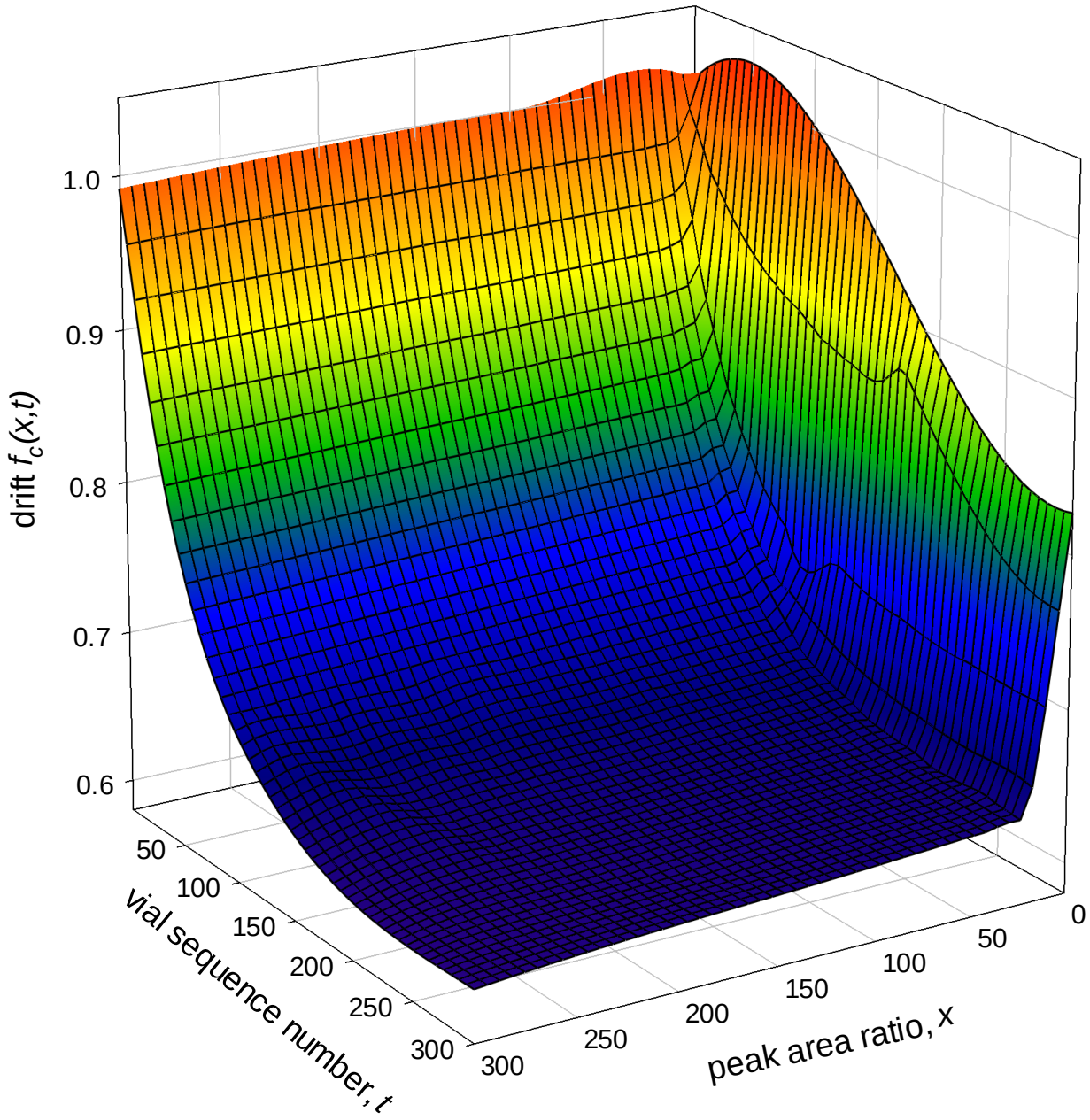

Figure A1.4. Drift correction function $f_c(x,t)$. Note rapid change with x and more gradual change with t.

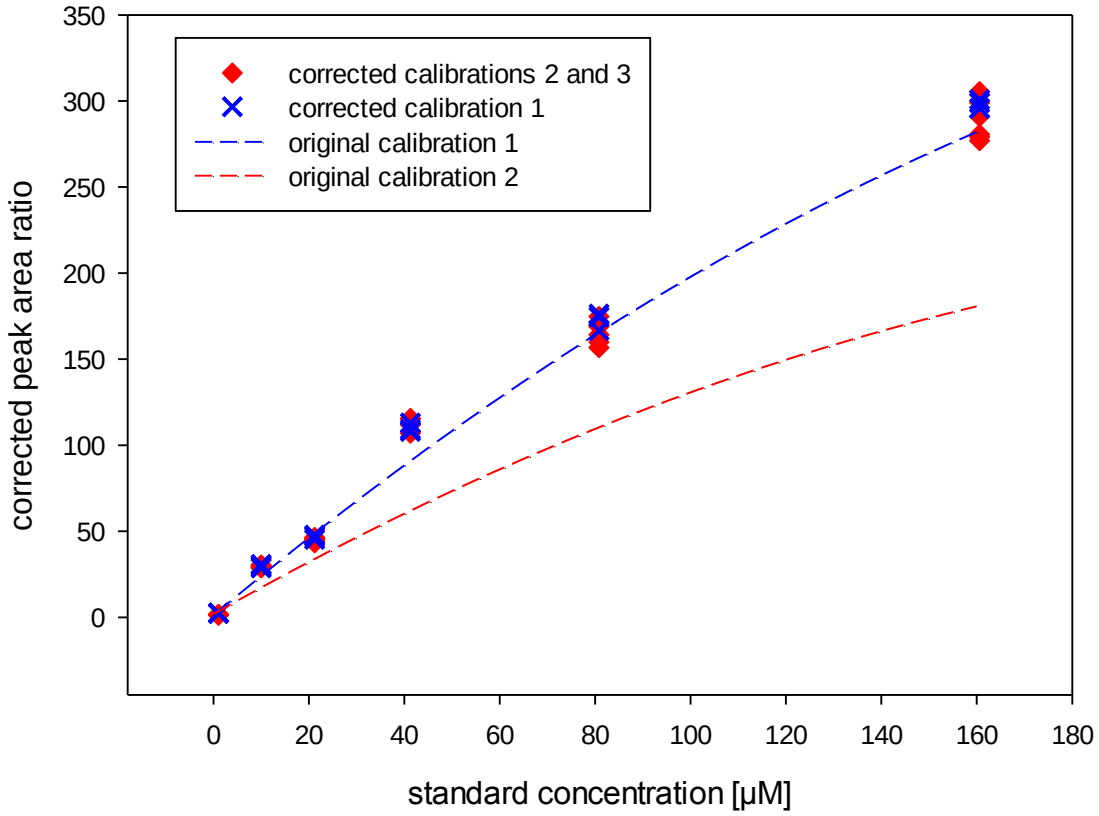

Figure A1.5. Drift-corrected AsB calibration functions. The original calibration curves are shown for comparison.

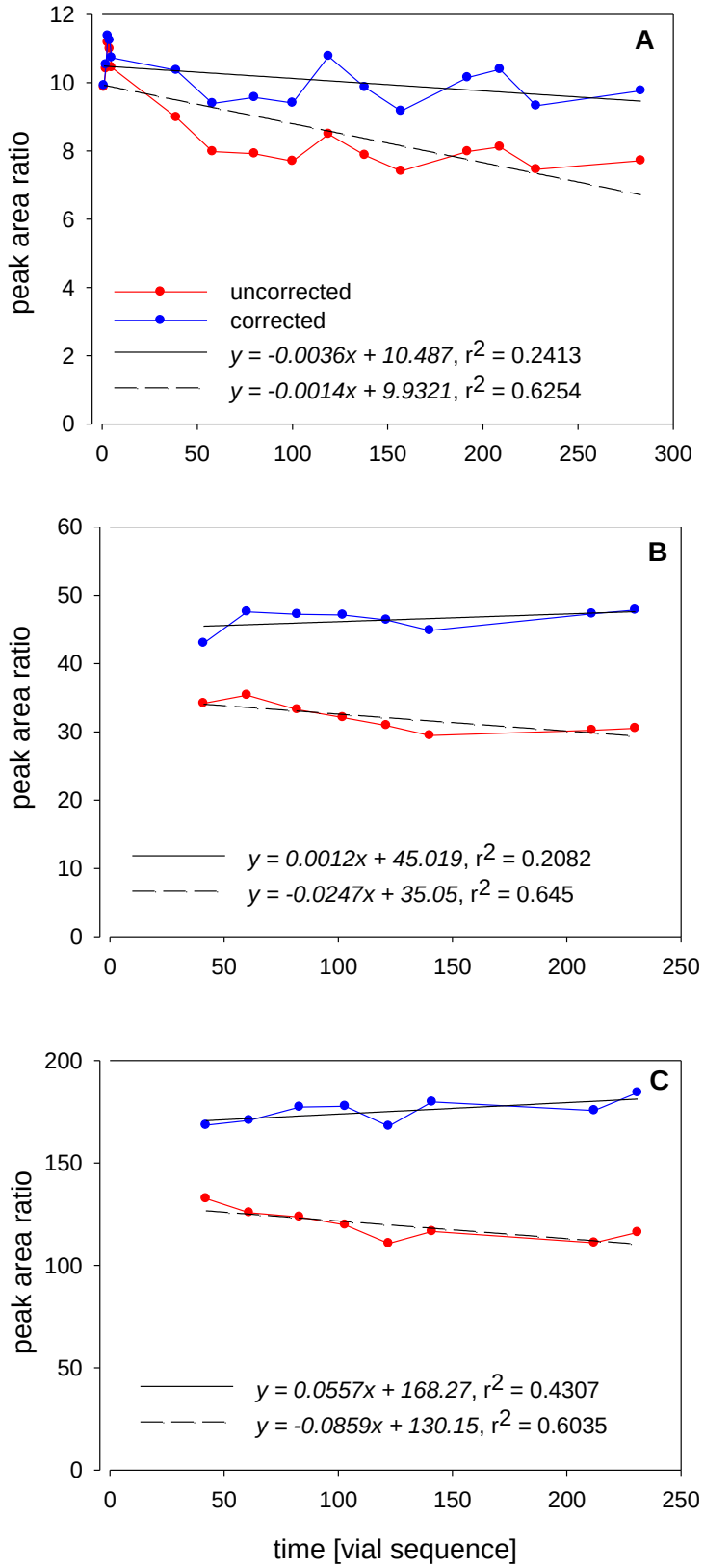

Figure A1.6. Uncorrected and drift corrected peak area ratios for AsB in low QC, 25 µM (A) low standard, 20 µM (B) and high standard, 80 µM (C).

DMSP drift correction in prey samples

The was a change in signal response between the first and later calibrations but there was no further drift between the second and third calibrations, so these data were pooled (Figure A1.7).

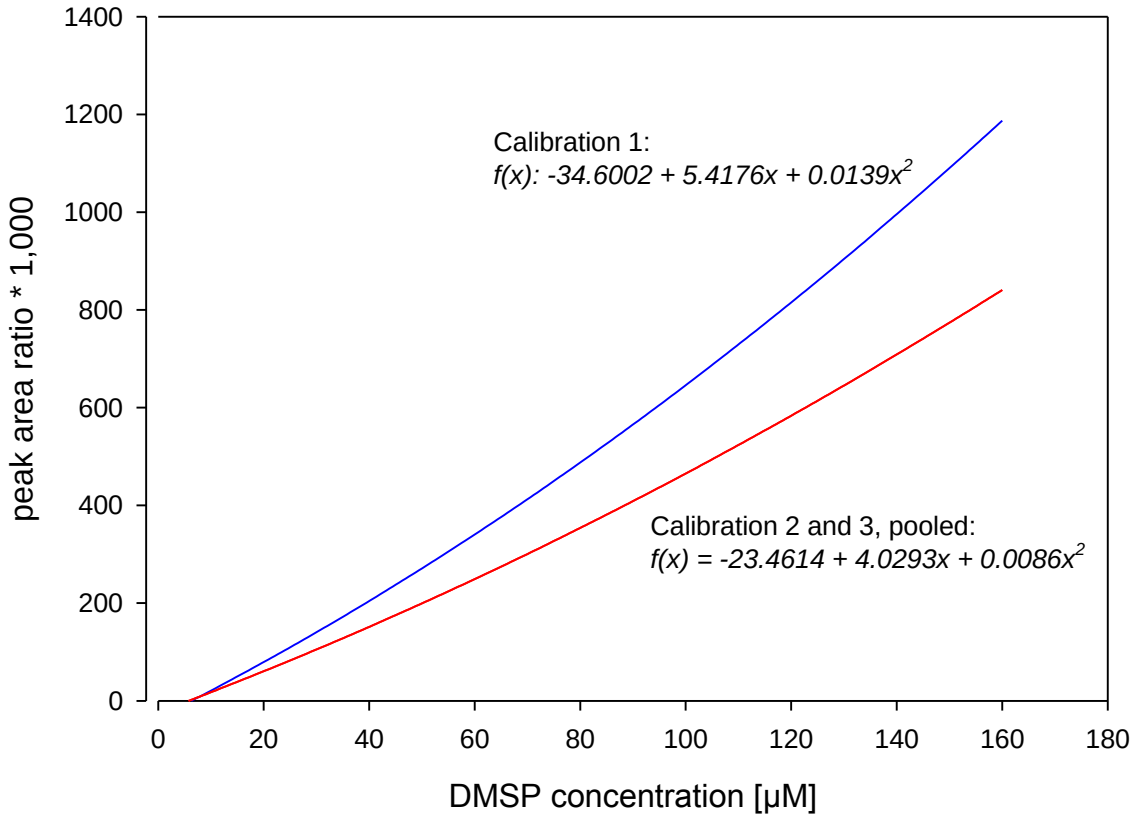

Figure A1.7. Change in DMSP signal response (external calibration function) between the initial (1) and later (2,3) calibrations.

The signal response was not linear but followed a second order curve (Figure A1.7). An exponential decay curve was modelled for each QC (25 and 80 µM) and standard (20 and 80 µM) separately to derive the initial signal value, and peak area ratios were normalised by dividing them by this initial value. An exponential decay function was then fitted to the pooled normalised data to derive an estimate of *k*, the decay constant for the relative change of signal with time (Figure A1.8).

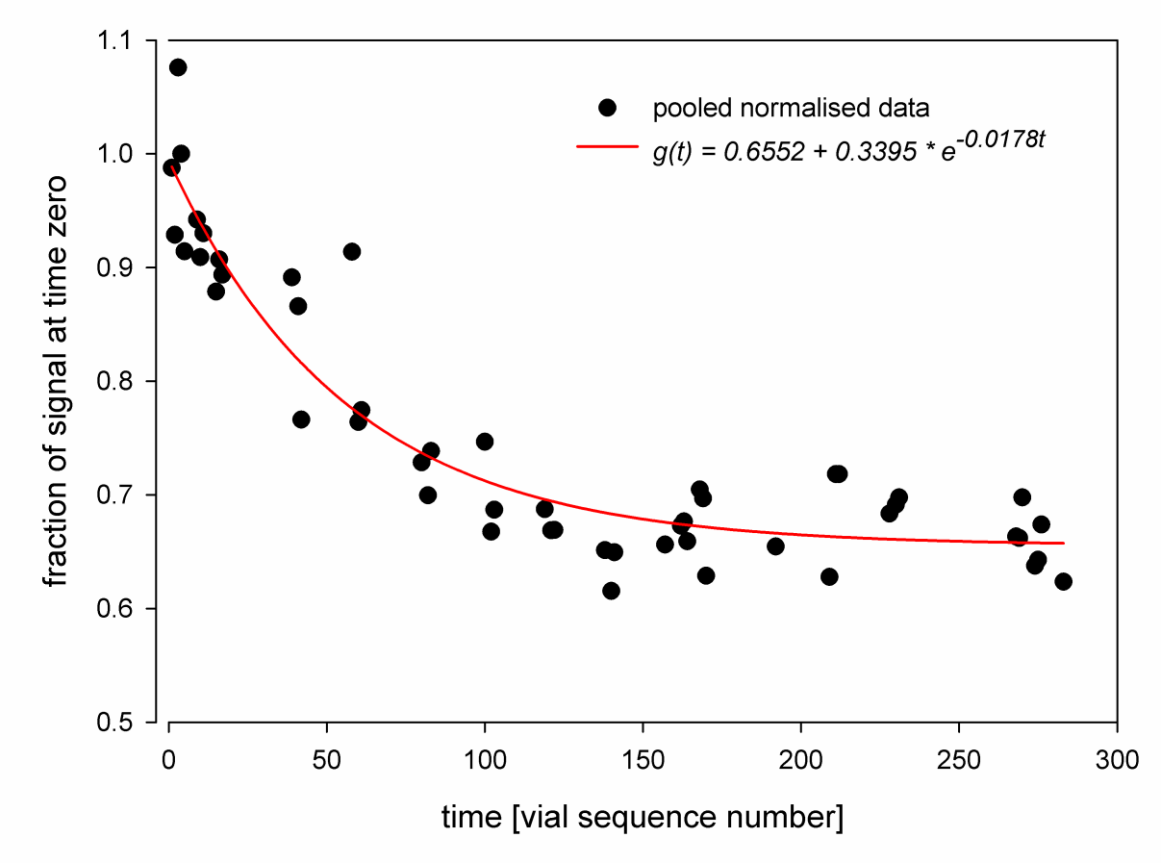

Figure A1.8. Pooled fractional drift in the normalised signal response of QC samples.

The exponential equation in Figure A1.8 was used as the drift correction factor where *t* is the vial sequence number. Measured peak area ratios for sample replicates were divided by the drift correction factor to obtain the corrected peak area ratio. Corrected peak area ratios for calibration standards were used to derive a pooled calibration function. For DMSP, the corrected pooled peak area ratios were separated into two plots; one with concentrations 1-40 µM (Figure A1.9a) and the second with concentrations 40-160 µM (Figure A1.9b).

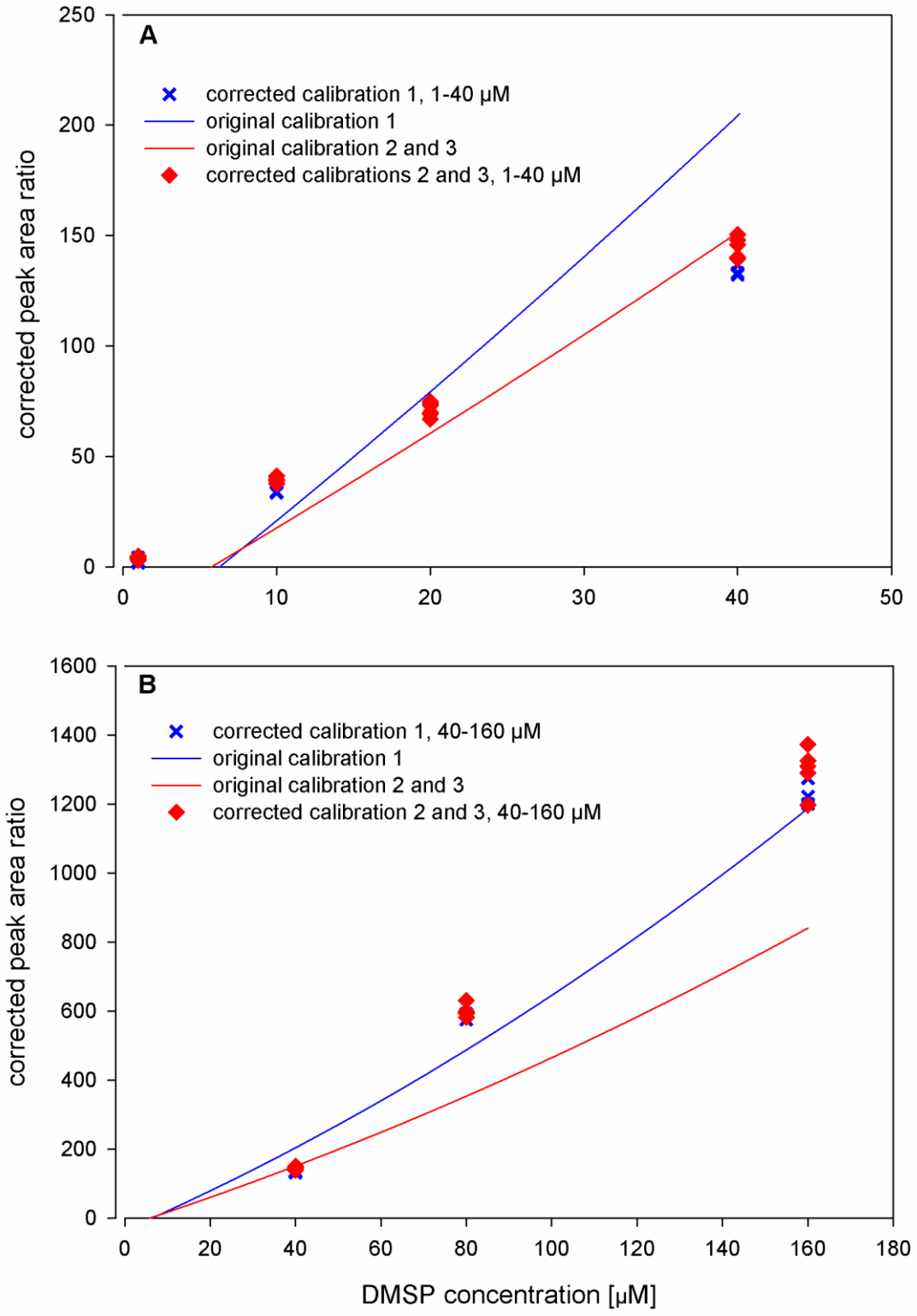

Figure A1.9. Drift corrected DMSP calibration functions. The calibration curve was split into two plots, 1-40 µM (A) and 40-160 µM (B). The original calibration curves are shown for comparison.

A weighted Demming linear regression equation (*Eq. 4*) fitted to corrected 1-40 µM pooled calibrations (Figure A1.9a) was used to correct the peak area ratio of QCs (Figure A1.10) and unknown samples that had a PAR below 150. An inverse second order equation (*Eq. 5*) fitted to corrected 40-160 µM pooled calibrations (Figure A1.9b) was used to correct the peak area ratio of QCs (Figure A1.10) and unknown samples that had a PAR above 150. *T* is the vial sequence number and *x* is peak area ratio:

$$f(x) = 3.717t - 0.498 \qquad \text{Eq. 4}$$

$$f(x) = 27.9660 + 0.0786t + 1.96e^{-5}t^{2} \qquad \text{Eq. 5}$$

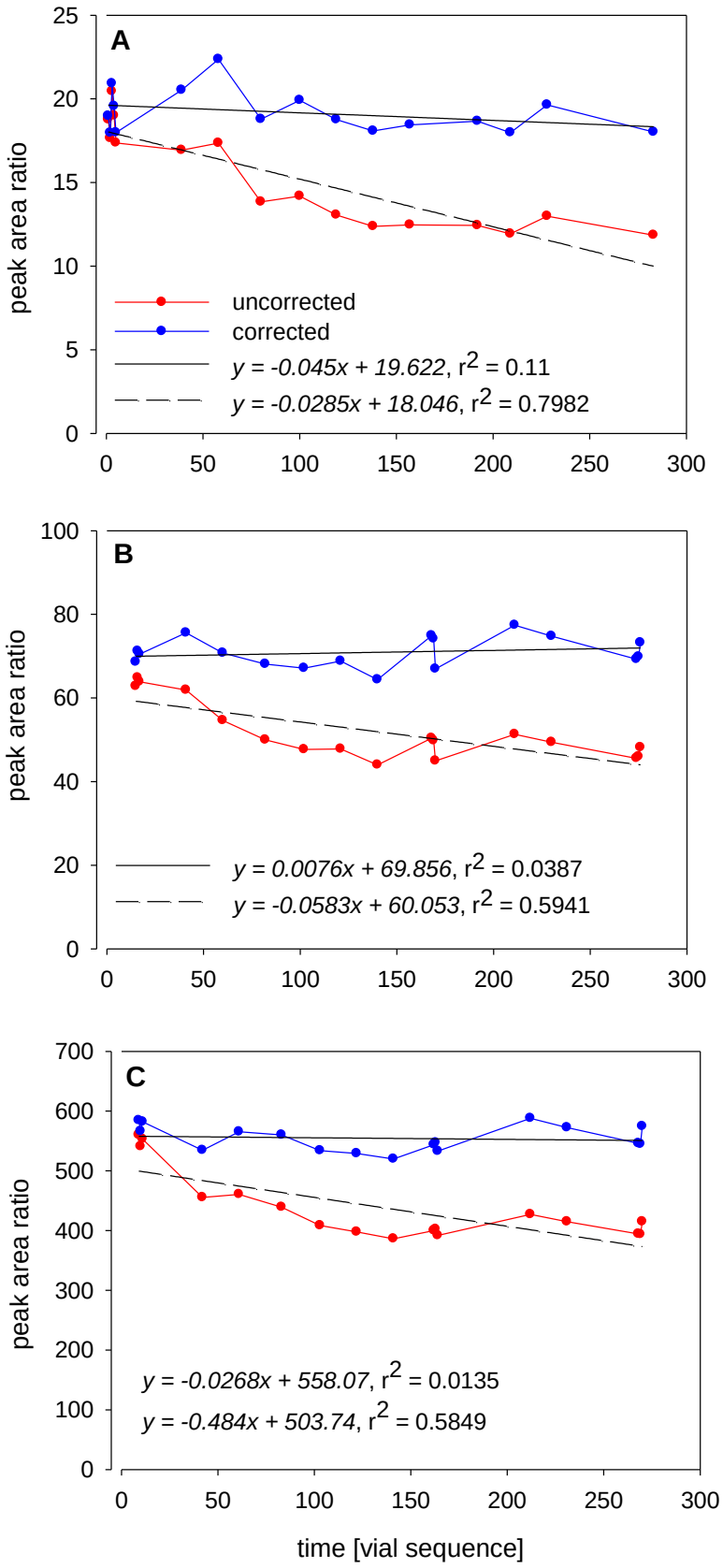

Figure A1.10. Uncorrected and drift corrected peak area ratios for DMSP in low QC, 25 µM (A) low standard, 20 µM (B) and high standard, 80 µM (C).

Arsenobetaine drift correction in serum

There was little drift between the first, second and third calibrations (Figure A1.11).

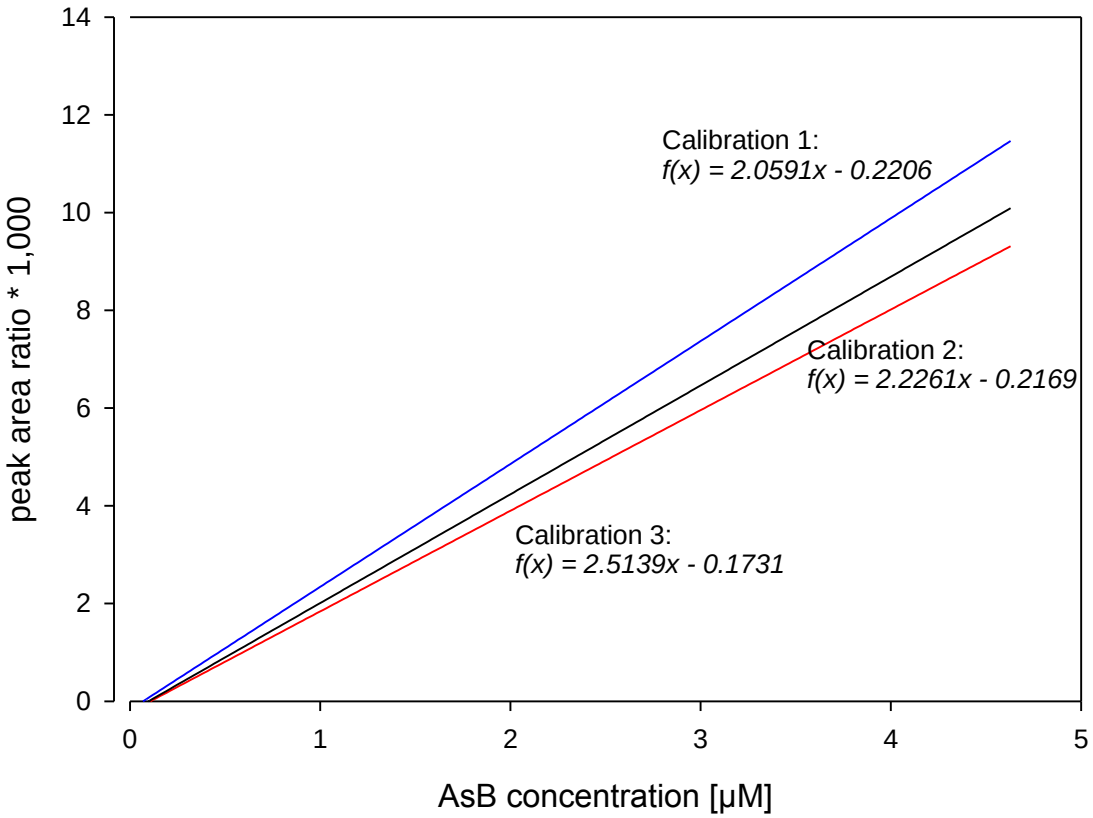

Figure A1.11. Change in signal response (external calibration function) between the initial (1) and later (2,3) calibrations.

An exponential decay curve was modelled for each QC (0.5 and 2 µM) and standard (0.1 and 2 µM) separately to derive the initial signal value, and peak area ratios were normalised by dividing them by this value. A Passing Bablock linear equation was fitted to the pooled normalised data as the drift in signal response was linear over time (Figure A1.12).

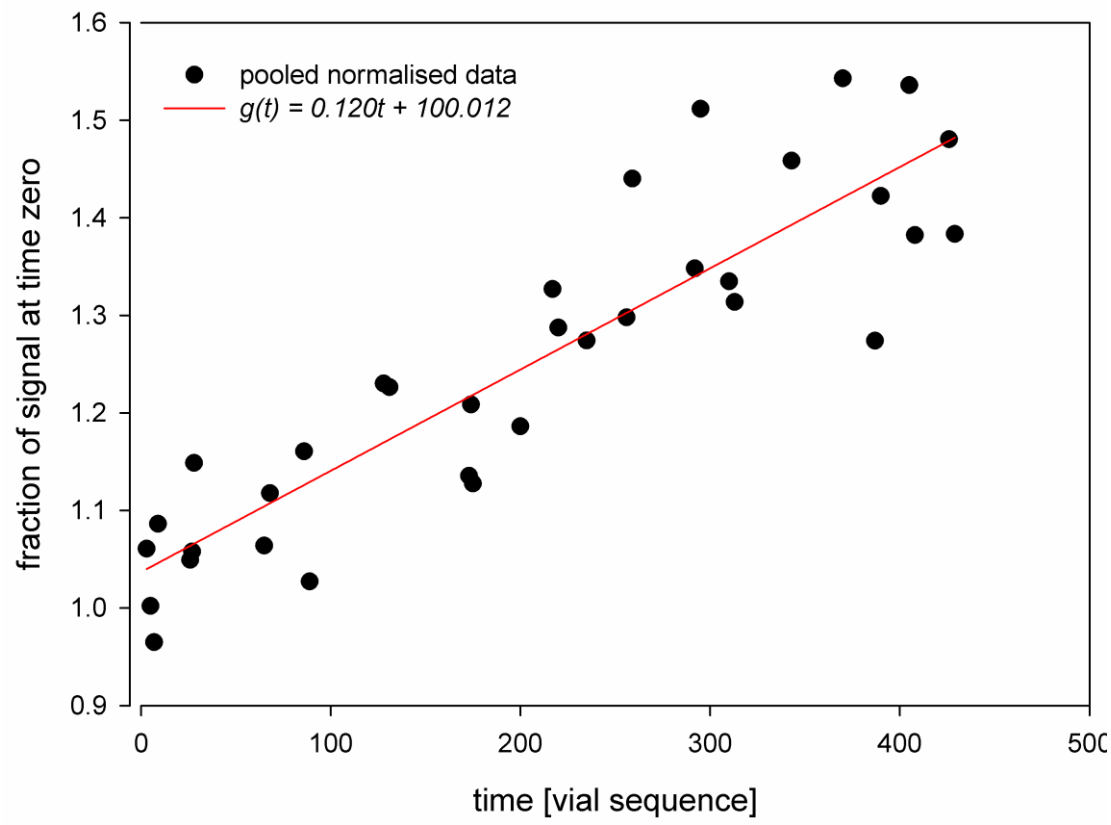

Figure A1.12. Pooled fractional drift in the normalised signal response of QC samples.

The linear equation in Figure A1.12 was used as the drift correction factor where *t* is the vial sequence number. Measured PAR for sample replicates were divided by the drift correction factor to obtain the corrected APR. Corrected PAR for calibration standards were used to derive a pooled calibration function (Figure A1.13).

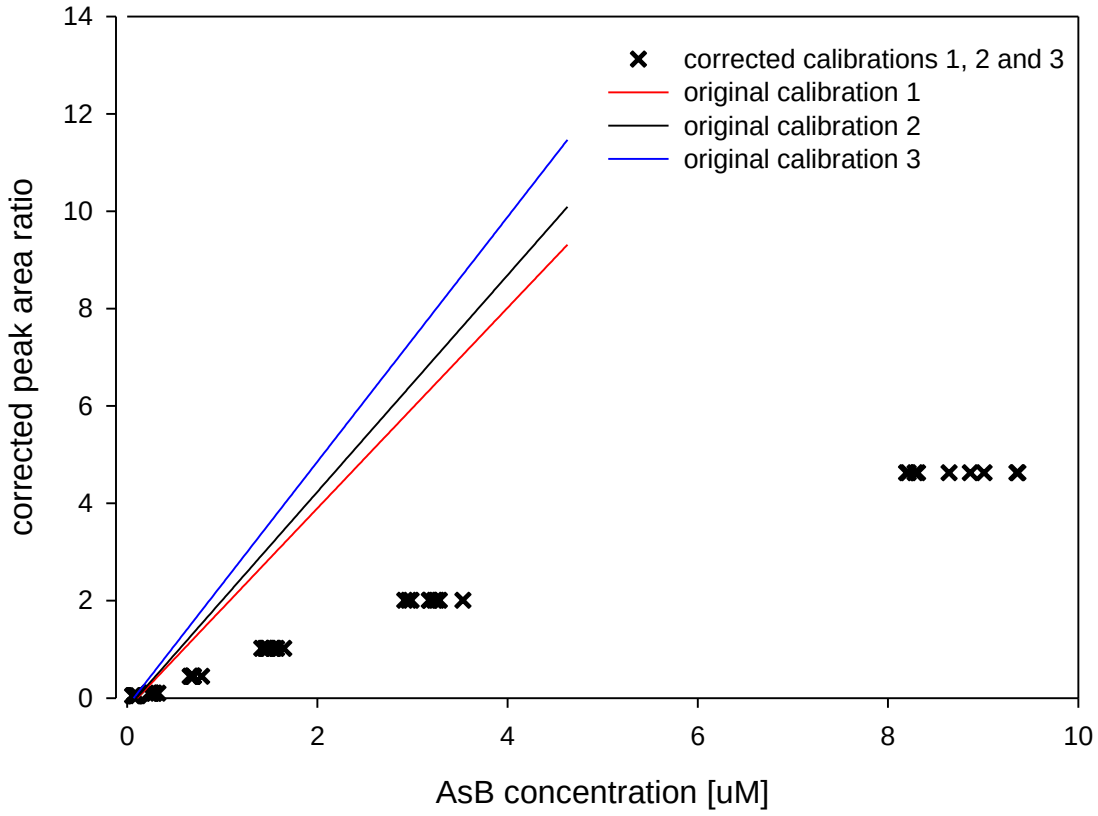

Figure A1.13. Drift corrected calibration functions. The original calibration curves are shown for comparison.

An inverse second order equation (*Eq. 6*) fitted to corrected pooled calibrations (Figure A1.13) was used to correct the PAR of QCs (Figure A1.14) and unknown samples. *T* is the vial sequence number and *x* is peak area ratio:

$$f(x) = -0.0503 + 0.733t - 0.0266t^2 \qquad Eq.\ 6$$

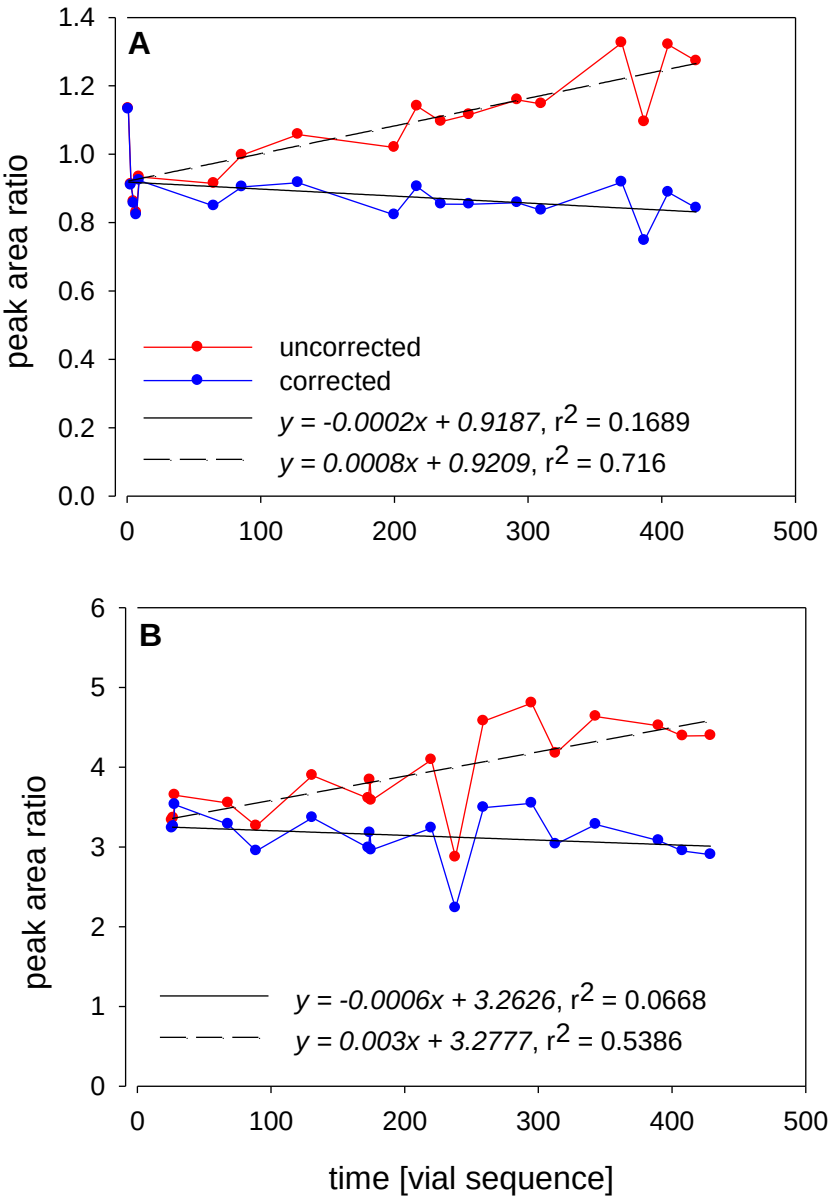

Figure A1.14. Uncorrected and drift corrected peak area ratios for AsB in low QC, 0.5 µM (A) and low standard, 2 µM (C).

TMAO drift correction in serum

There was a change in signal response between the first and later calibrations (Figure A1.15). The peak area of TMAO was used for calibration instead of the peak area ratio between TMAO: D_9-TMAO because it gave a better fit to the data.

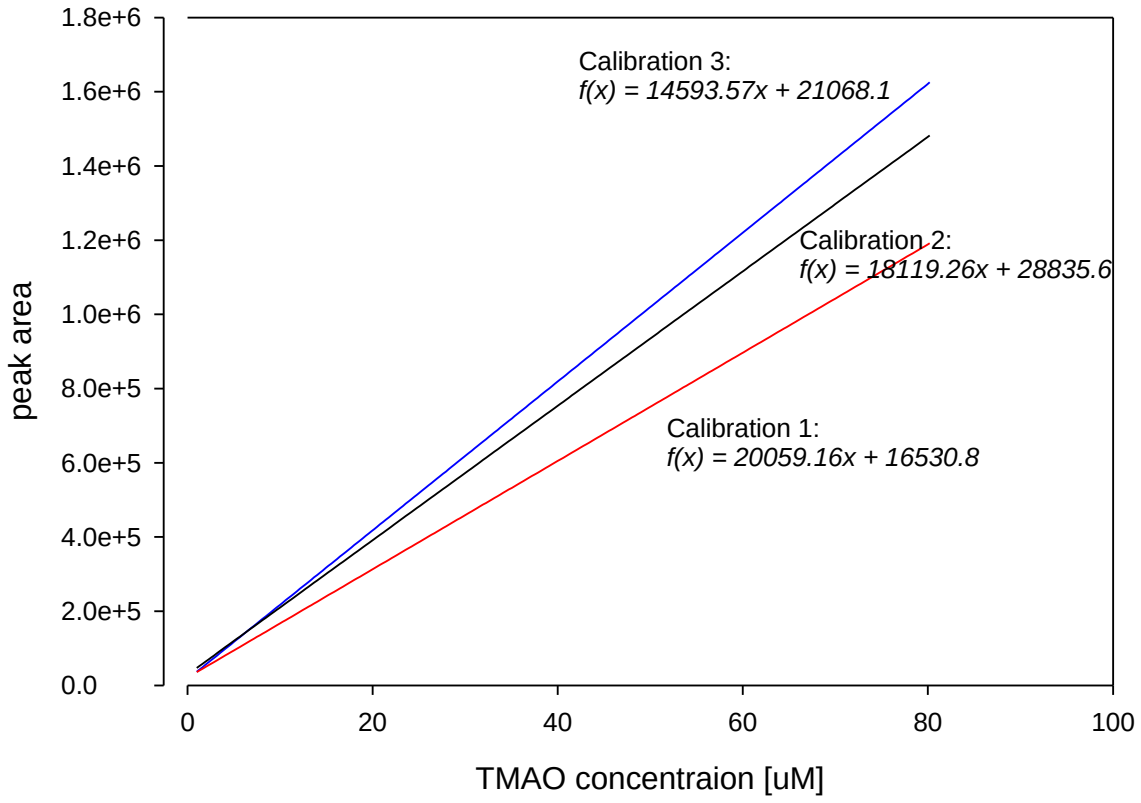

Figure A1.15. Change in signal response (external calibration function) between the initial (1) and later (2,3) calibrations.

An exponential decay curve was modelled for each QC (5 and 45 µM) and standard (1 and 20 µM) separately to derive the initial signal value, and peak area ratios were normalised by dividing them by this value. An exponential decay function was then fitted to the pooled normalised data to derive an estimate of *k*, the decay constant for the relative change of signal over time (Figure A1.16).

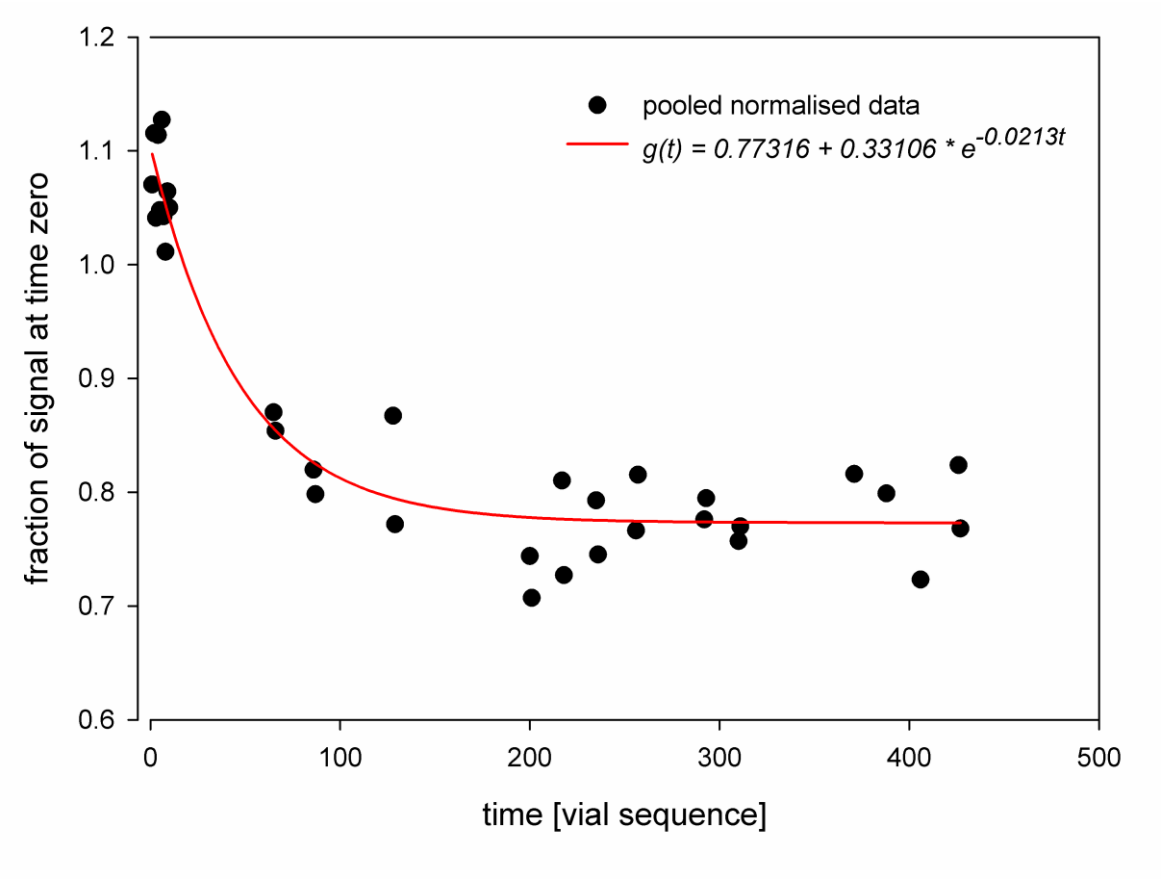

Figure A1.16. Pooled fractional drift in the normalised signal response of QC samples.

The exponential equation in Figure A1.16 was used as the drift correction factor where *t* is the vial sequence number. Measured peak areas for sample replicates were divided by the drift correction factor to obtain the corrected peak area. Corrected peak area for calibration standards were used to derive a pooled calibration function (Figure A1.17).

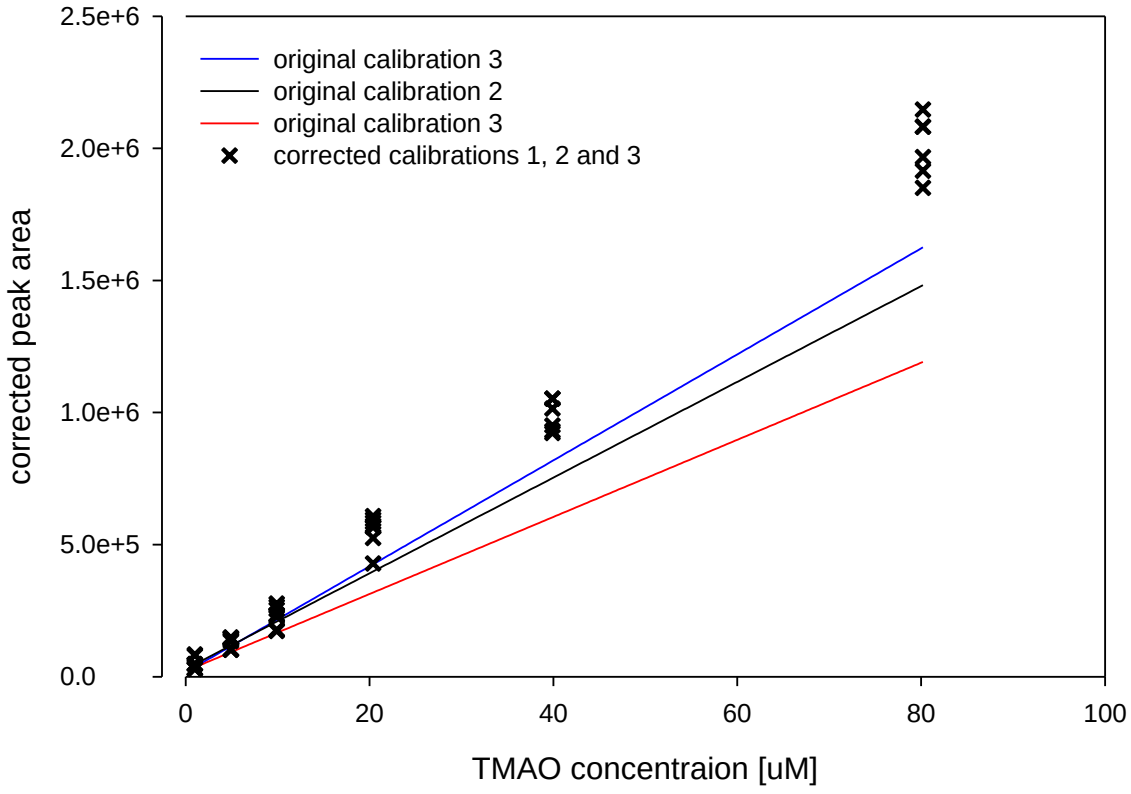

Figure A1.17. Drift-corrected TMAO calibration functions. The original calibration curves are shown for comparison.

A weighted Deming linear regression (*Eq. 7*) fitted through the pooled corrected calibrations (Figure A1.17) was used to correct the peak area of QCs (Figure A1.18) and unknown samples. *T* is the vial sequence number and *x* is peak area:

$$f(x) = 24558t + 10555 \qquad Eq.\ 7$$

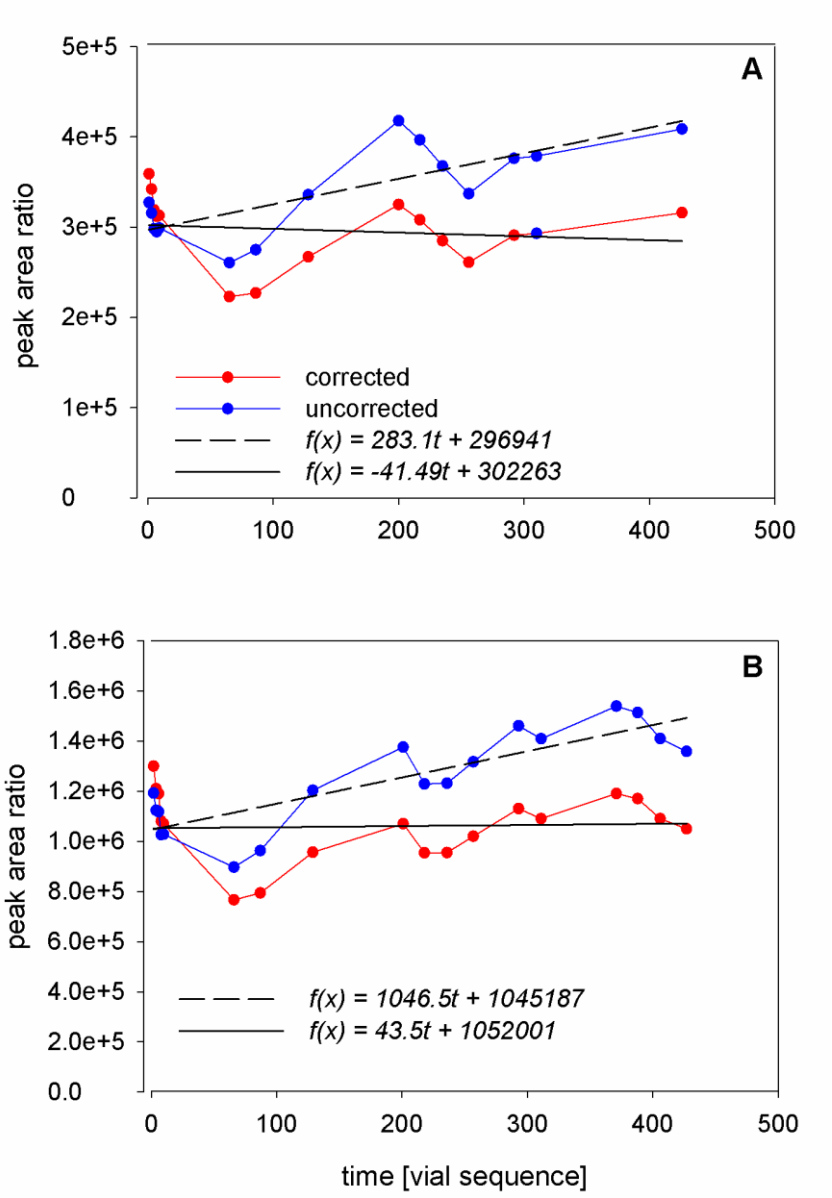

Figure A1.18. Uncorrected and drift corrected peak area ratios for TMAO in low QC, 5 µM (A) and high QC, 45 µM (C).

Appendix B

Table B1.1. Overview of fish and invertebrates collected from McMurdo Sound and the Ross Sea, Antarctica.

Species	Collection Date	Sampling site[1]	Latitude (S)[2]	Longitude (E)	Gear Type[3]	Depth (m)	Tissue	*n*
Nototheniidae								
Dissostichus mawsoni	November 2007	West of Turtle Rock, MS	77.72307*	166.79908*	Hook & line	unknown	White muscle fillet	1
Lepidonotothen squamifrons	March 10, 2008	RS, Admiralty seamounts	66.9925 (start) 66.9795 (finish)	170.877 (start) 170.8315 (finish)	ORH	566-920	Whole	5
Pagothenia borchgrevinki	December 1, 2006	MS, Arrival Heights	77.81271*	166.63191*	Hook & line	unknown	Whole	6
Pagothenia borchgrevinki	December 2, 2007	MS, Cape Evans	77.64066*	166.39653*	Hook & line	unknown	Whole	4
Pleuragramma antarcticum	February 9, 2008	central Ross Sea	73.124 73.133	174.320 174.303	MWT	320	Whole	15
Trematomus bernacchii	November 21, 2006	MS, Inaccessible Island	77.66145*	166.40214*	Hook & line	unknown	Whole	6
Trematomus bernacchii	October 30, 2007	MS, Winters Quarter Bay	77.84699*	166.64639*	Hook & line	unknown	Whole	6
Trematomus bernacchii	December 3, 2007	MS, Cape Evans	77.64066*	166.39653*	Hook & line	Unknown	Whole	5
Trematomus bernacchii	October 11, 2007	MS, salt water intake jetty	77.85138*	166.66083*	Hook & line	unknown	Whole	1
Trematomus eulepidotus	February 11, 2008	southern Ross Sea	74.58167 74.5805	170.24983 170.293	ORH	285	Whole	5
Trematomus hansoni	December 18, 2007	MS, salt water intake jetty	77.85138*	166.6608*	Hook & line	~ 15	Whole	7
Trematomus lepidorhinus	February 23, 2008	central Ross Sea	71.9385 71.9213	173.3023 173.3003	ORH	1431-1658	Whole	5
Trematomus pennellii	October 11, 2007	MS, salt water intake jetty	77.8513*	166.6608*	Hook & line	unknown	Whole	2
Trematomus scotti	February 23, 2008	southeastern Ross Sea	76.59367 76.59883	176.8275 176.7545	ORH	366-369	Whole	5
Bathylagidae							Whole	
Bathylagus antarcticus	February 26, 2008	central Ross Sea	71.343 71.313	174.8267 174.9168	MWT	50-1010		5
Myctophidae							Whole	
Electrona antarctica	February 23, 2008	RS, Scott Islands	68.13617 68.11433	180.6563 180.5752	MWT	10-781		30
Electrona carlsbergi	March 2, 2008	RS, Scott Islands	71.98183 72.0283	173.3927 173.3958	MWT	10-781	Whole	15
Gymnoscopelus braueri	March 12, 2008	RS, Admiralty seamounts	66.9061 66.9945	171.0615 171.089	MWT	50-1032	Whole	5

Species	Collection Date	Sampling site[1]	Latitude (S)[2]	Longitude (E)	Gear Type[3]	Depth (m)	Tissue	*n*
Gymnoscopelus nicholsi	March 11, 2008	RS, Admiralty seamounts	66.875 66.789	171.2865 171.2365	MWT	50-1004	Whole	5
Mollusca								
Kondakovia longimana	Feb-March 2008	RS, Scott Islands	68.271	181.106	MWT	unknown	Freeze dried sample	1
Kondakovia longimana	Feb-March 2008	RS, Admiralty seamounts	66.971	170.714	ORH	unknown	Freeze dried	2
Mastigoteuthis psychrophila	Feb-March 2008	RS, Admiralty seamounts	75.632	169.883	ORH	unknown	Freeze dried sample	1
Psychroteuthis glacialis	March 11, 2008	RS, Admiralty seamounts	66.832	171.262	MWT	50-1004	Freeze dried sample	1
Psychroteuthis glacialis	March 10, 2008	RS, Admiralty seamounts	66.989	170.854	ORH	445-455	Freeze dried sample	1
Psychroteuthis glacialis	Feb-March 2008	central Ross Sea	72.371	175.503	ORH	unknown	Freeze dried sample	3
Pareledone turqueti	early Dec 2007	MS, west of Turtle Rock	77.7439*	166.7138*	Baited trap	450-500	Whole	4
Pareledone 1 *(cf. albimaculata)*	February 9, 2008	central Ross Sea	73.128	174.312	ORH	320	Freeze dried sample	3
Pareledone 2 *(cf. aequipapillae)*	Feb-March 2008	southern Ross Sea	75.616	167.316	Trawl	unknown	Freeze dried sample	1
Pareledone 2 *(cf. aequipapillae)*	February 17, 2008	southeastern Ross Sea	76.596	176.791	ORH	366-369	Freeze dried sample	1
Pareledone 3 *(cf. charcoti)*	Feb-March 2008	RS, unknown	71.740	171.167	Trawl	unknown	Freeze dried sample	1
Pareledone 3 *(cf. charcoti)*	Feb-March 2008	RS, unknown	71.740	171.148	Trawl	unknown	Freeze dried sample	1
Pareledone 3 *(cf. charcoti)*	Feb-March 2008	RS, unknown	71.740	171.644	Trawl	unknown	Freeze dried sample	1
Pareledone 4 (cf. *turqueti*)	Feb-March 2008	southern Ross Sea	75.632	169.883	Trawl	unknown	Freeze dried sample	1
Pareledone 4 (cf. *turqueti*)	February 11, 2008	southern Ross Sea	74.589	170.272	Trawl	285	Freeze dried sample	1
Pareledone 4 (cf. *turqueti*)	Feb-March 2008	RS, Scott Islands	67.830	180.407	Trawl	unknown	Freeze dried sample	1
Pareledone 4 (cf. *turqueti*)	Feb-March 2008	southern Ross Sea	75.616	167.316	Trawl	unknown	Freeze dried sample	1
Pareledone 4 (cf. *turqueti*)	February 17, 2008	southeastern Ross Sea	76.596	176.791	ORH	366-369	Freeze dried sample	1
Pareledone 4 (cf. *turqueti*)	Feb-March 2008	southern Ross Sea	74.718	167.019	Trawl	unknown	Freeze dried sample	1
Pareledone 4 (cf. *turqueti*)	February 25, 2008	central Ross Sea	71.872	174.071	ORH	1954-1990	Freeze dried sample	1
Marseniopsis mollis	December 2007	MS, Cape Armitage	77.86166*	166.67916*	Baited trap	< 50	Whole	8
Crustacea								
Lysianassid amphipod	December 16, 2007	MS, Hutton Cliffs	77.72307*	166.79908*	Baited trap	< 40	Whole	~400
Euphausia crystallorophias	Feb-March 2008	southeastern Ross Sea	76.596*	176.791*	Trawl	unknown	Whole	~100
Euphausia crystallorophias	Feb-March 2008	central Ross Sea	74.644	169.018	MWT	445-456	Whole	~100
Euphausia superba	Feb-March 2008	central Ross Sea	71.343	174.826	MWT	50-1010	Whole	~100
Glyptonotus antarcticus	December 2007	MS, Cape Armitage	77.86166*	166.67916*	Divers	< 50	Whole	5
Echinodermata								
Odontaster validus	December 2007	MS, Cape Armitage	77.86166*	166.67916*	Baited trap	< 50	Whole	6
Staurocucumis turqueti	December 7, 2007	MS, Cape Armitage	77.86166*	166.67916*	Baited trap	< 50	Whole	4
Sterechinus neumayeri	December 2007	MS, Cape Armitage	77.86166*	166.67916*	Baited trap	< 50	Whole	7
Other Invertebrates								

Species	Collection Date	Sampling site[1]	Latitude (S)[2]	Longitude (E)	Gear Type[3]	Depth (m)	Tissue	*n*
Flabelligera mundata	October 11, 2007	MS, Winters Quarter Bay	77.846992*	166.64639*	Divers	< 50	Whole	
Parborlasia corrugatus	December 16, 2007	MS, Hutton Cliffs	77.72307*	166.79908*	Baited trap	< 50	Whole	5
Cnemidocarpa verrucosa	December 2007	MS, Cape Armitage	77.86166*	166.67916*	Baited trap	< 50	Whole	13

[1] Sampling site: MS = McMurdo Sound, RS = Ross Sea

[2] Latitude and longitude start/finish locations for samples collected in the RS provided by Matt Pinkerton (NIWA). * = estimated.

[3] Gear type: MWT = mid water trawl, ORH = orange roughy bottom trawl, used mainly on seamounts and for collecting demersal fish, trawl = specimen taken at a core sampling site where a number of different trawl types were employed (Hanchett 2008).

Appendix C

Female Weddell seals without TDR data

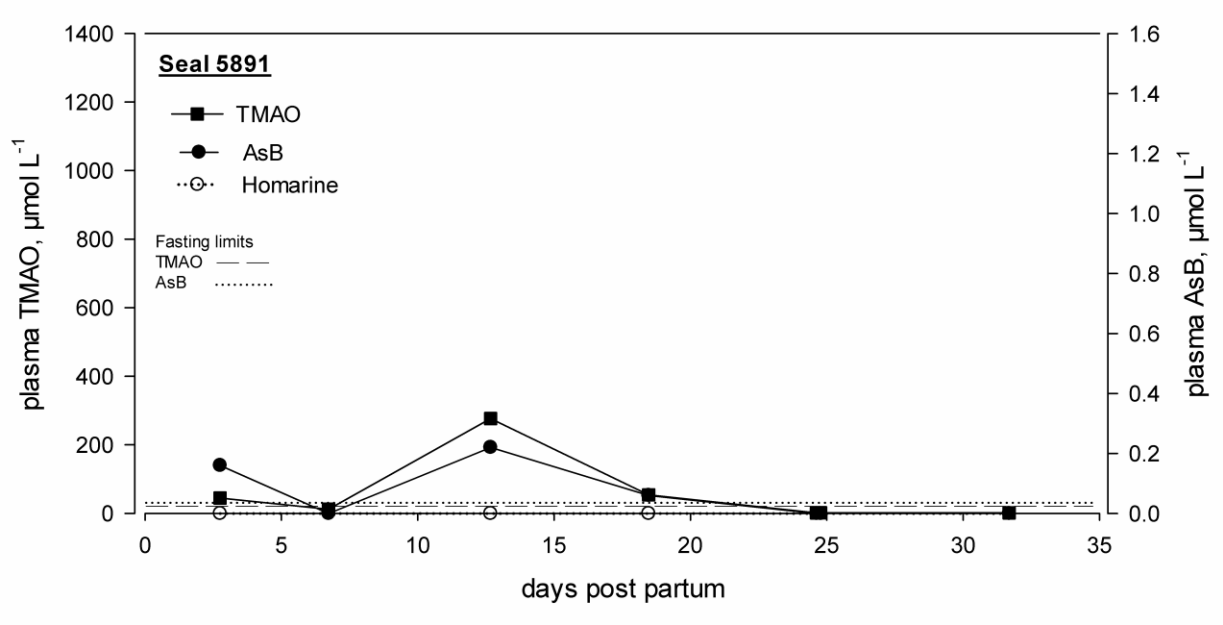

Figure C1.1. Plasma biomarker concentrations in female 5891 during the lactation period.

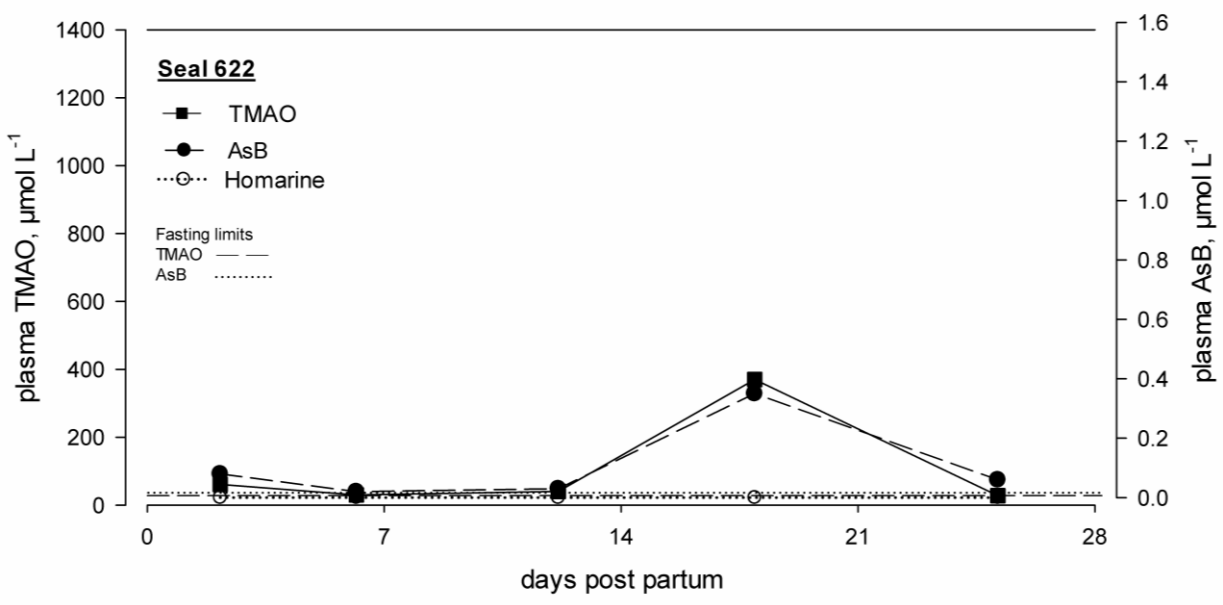

Figure C1.2. Plasma biomarker concentrations in female 622 during the lactation period.

Female Weddell seals not feeding during the study period

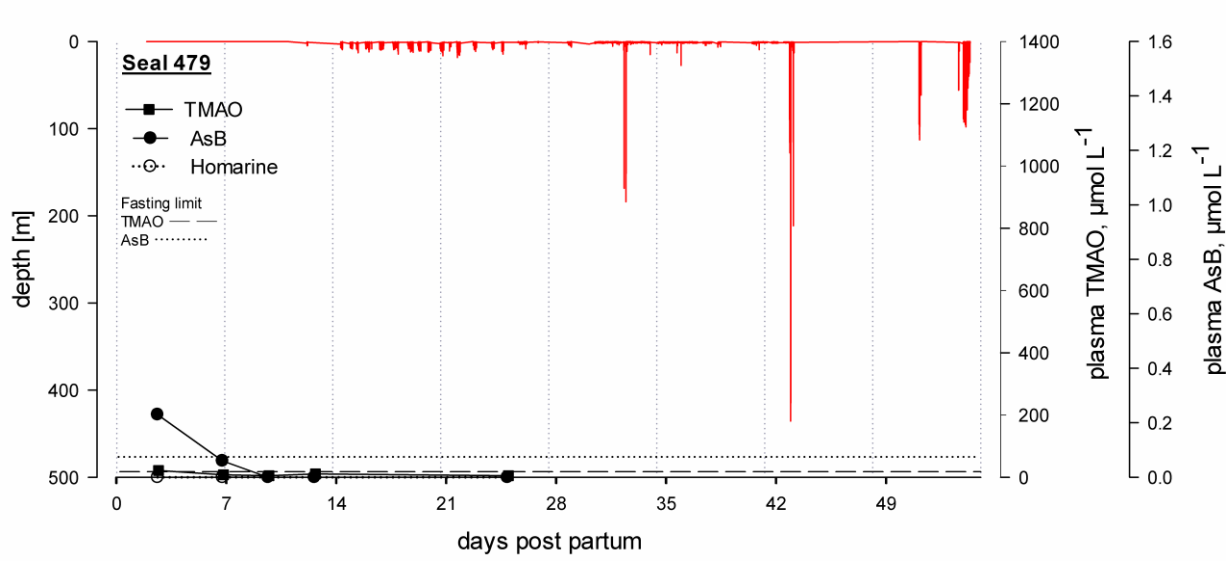

Figure C1.3. Comparison of dive activity with plasma biomarkers in female 479 over the lactation period.

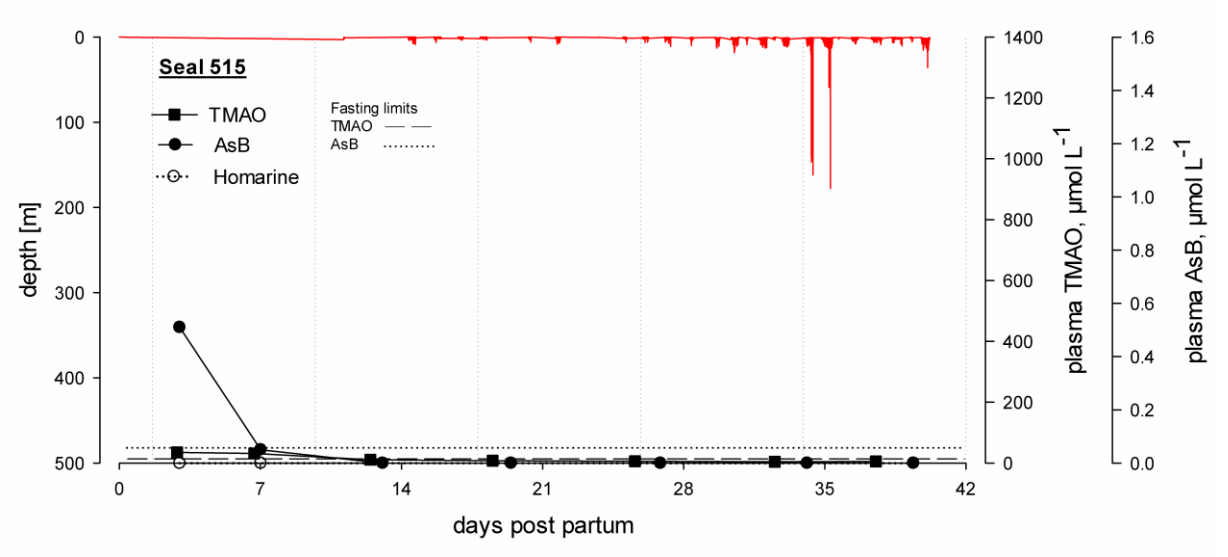

Figure C1.4. Comparison of dive activity with plasma biomarkers in female 515 over the lactation period.